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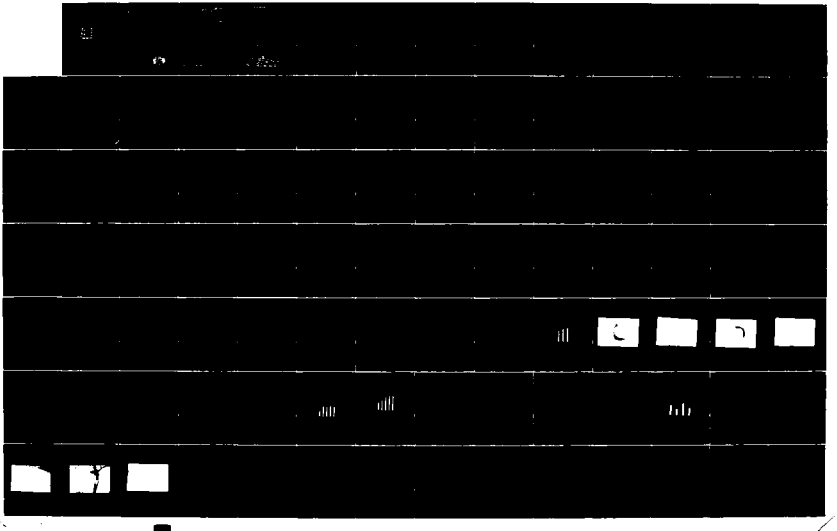

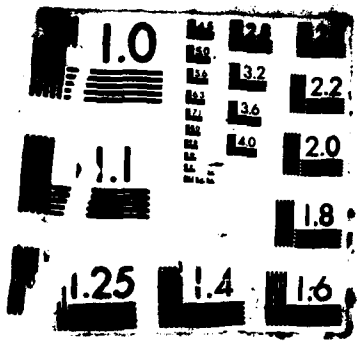
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DERMAL INFLUENCE ON EPIDERMAL RESURFACING DURING THE REPAIR OF SPLIT THICKNESS WOUNDS

ANNUAL/FINAL REPORT

Patricia A. Hebda, Ph.D.
William H. Eaglstein, M.D.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The purpose of this study was to evaluate dermal-epidermal interrelationships during wound repair by assessing the effect on wound healing of factors derived from platelets. Two methods were used to approach the problem: 1) an in vitro epithelialization model in which small sections of partial-thickness pig skin (explants) were grown in medium containing fetal bovine serum and/or a platelet homogenate or purified growth factors, and epidermal cell outgrowth from the explants was measured; 2) a wound healing animal model in which partial-thickness

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excisional wounds made on the backs of young domestic pigs were treated with platelet preparations or purified growth factors then evaluated for re-epithelialization and dermal collagen biosynthesis. Our results are as follows:

1. In Explant Cultures:

   a. Platelet Homogenate Fraction (PHF) initiated epidermal cell outgrowth and supported explant viability without serum. The factor producing this effect has an apparent molecular weight larger than 30 K, and is heat stable (100°C for 5 min).

   b. PHF stimulated the rate of epidermal outgrowth. The factor producing this effect has an apparent molecular weight less than 30 K and is heat labile (100°C for 5 min).

   c. Commercial Platelet-derived Growth Factor (PDGF) without serum did not support explants. With serum it had a slight stimulatory effect on the rate of outgrowth.

   d. Transforming Growth Factor-beta (TGF-beta) produced earlier initiation of outgrowth and stimulated the rate of outgrowth during the migratory phase of culture.

   e. Epidermal Growth Factor (EGF) increased explant viability and stimulated the rate of outgrowth during the mitotic phase of culture.

   f. The activities in PHF are distinguishable from PDGF, TGF-beta and EGF, based on molecular weight, heat sensitivity and biologic activity.

2. In Wound Healing:

   a. PHF in a variety of vehicles was tested for stimulation of epidermal wound healing (epithelialization) and dermal wound healing (collagen synthesis). PHF in 2.5% carboxymethyl-cellulose produced faster epithelialization compared to vehicle alone, but did not enhance dermal collagen synthesis.

   b. TGF-beta in white petrolatum produced faster epithelialization and possibly increased dermal collagen synthesis.

   c. The results of these wound healing studies warrant further investigation.

Our study is the first to demonstrate a stimulatory effect of platelet-derived factors on epidermal cells. Our conclusions are that platelet components stimulate epidermal cell viability and outgrowth in explant cultures. We recommend further exploration of these factors. The factors should be purified from platelets, compared with known growth factors, and evaluated first in vitro with epidermal explant and cell culture systems. Active factors should be tested alone and in combination in animal wound healing trials. Finally, an effective treatment should be evaluated in a clinical study with human subjects for effects on acute wounds, burn wounds and chronic ulcers.

B

ABSTRACT

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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PROBLEM

This study focuses on epidermal and dermal interrelationships during wound repair. Blood platelets contain growth factors for dermal fibroblasts. We have shown that platelets also contain a factor or factors that affect epidermal cells in explant cultures. We characterized the effects of platelets on the outgrowth of epidermal cells in a skin explant culture model and on the rate of epidermal and dermal wound healing in an animal model. The goals of this study were directed toward obtaining knowledge of the wound healing process and the role of platelets in order to develop more effective ways to treat skin injury.

INTRODUCTION

The Skin

The skin is a two-layered covering resting on subcutaneous fat. The outer, highly cellular, thin epidermis is in contact with the dermis by way of multiple, interpapillary ridges (Krizek et al., 1968). These ridges result in increased surface contact between the two layers; they provide much of the resistance of normal skin to tangential stress. The epidermis and the dermis are separated by a continuous acellular basement membrane. The innermost portion of the epidermis is the basal layer containing the cells destined to become the keratinous outer stratum corneum. Cells generated from the basal layer are gradually extruded towards the surface as they undergo differentiation, forming first the stratum spinosum (Krizek et al., 1968), characterized by prominent interlocking cell wall projections which further aid the skin's ability to withstand shearing forces. The next layer is the stratum granulosum which merges imperceptibly with the stratum lucidum. These layers are involved with heat regulation and keratinocytes in these two layers synthesize the waterproof fibrous protein keratin. The keratinocytes mature and die, leaving a keratin sheath which makes up the outermost layer, the stratum corneum. The epidermis, especially the stratum corneum, is the body's primary defense against penetration by noxious environmental elements and also prevents fluid loss.

The underlying dermis, which is primarily collagen, elastin and proteoglycans, is 20 to 30 times thicker than the epidermis in human skin and contains the nervous, vascular, lymphatic and supporting structures of the skin, as well as harboring the epidermal appendages. Fibroblasts produce the fibrous proteins, collagen and elastin, which

give skin its strength (Gould, 1968). Mast cells containing histamine and heparin produce the proteoglycans (ground substance) (Grant and Prockop, 1972) which form the interfibrillary matrix of the dermis. Tissue macrophages are distributed around blood vessels and hair follicles (Pillsbury et al., 1965a). The appendages of human skin are the hair follicles and their associated sebaceous glands, the eccrine sweat glands which enter through interpapillary ridges, and the apocrine glands located primarily in the axillary and inguinal regions (Pillsbury et al., 1965b).

Wound Healing

The morphologic aspects of the wound healing process have been extensively studied. Ideally, the healing process will restore normal structures and may be considered regeneration; in most cases, it does not achieve the original state but repairs the defect. Repair occurs in dermal healing; collagen is produced to fill in the defect and restore the contour while angiogenesis restores the blood supply system. Some scarring almost always occurs with repair. Sometimes the repair process is aberrant and excess collagen results in keloids or hypertrophic scarring. Epidermal healing is closer to regeneration because most of the original structures are restored, by a sequence of migration, mitosis and maturation of epidermal cells.

In full thickness wounds, contraction accounts for a portion of the healing process. In partial thickness wounds, healing involves both the dermis and the epidermis, but contraction does not occur. While dermal fibroblasts are producing collagen, epidermal cells from the wound margins and in remaining epidermal appendages form a new surface (re-epithelialize) to cover the wound bed by undergoing migration,

mitosis and maturation. Dermal and epidermal healing occur simultaneously, but it is not yet known how these events are interrelated.

Wound Healing and Platelet-Derived Growth Factor

The first responses to excisional skin injury are bleeding, coagulation and inflammation. Blood enters the wound, a clot is formed by activated platelets and fibrin, and specific blood components including platelet-derived factors are released at the site of injury. Blood-derived growth factors were first proposed by Balk (1971). He demonstrated that nontransformed chick embryo fibroblasts grow well in medium with serum, but only virally transformed fibroblasts grow in plasma-supplemented medium. Ross et al. (1974) and Kohler and Lipton (1974) showed that platelets are the source of most of the mitogenic activity of serum. These studies led to the hypothesis that platelets activated at the site of vascular injury release components that trigger the proliferative response (Ross et al., 1974; Harker et al., 1976). Studies of this hypothesis resulted in the isolation and characterization of Platelet- derived Growth Factor (PDGF) which is a potent mitogen for mesenchymal cells, including smooth muscle cells and dermal fibroblasts both key participants in wound healing. PDGF is a heat stable, cationic, reducible protein approximately 30 K (Westermark and Wasteson, 1983; Ross and Vogel, 1978). PDGF is localized in the alpha granules of platelets and is released during platelet activation. It has many biological effects on connective tissue cells including stimulation of chemotactic activity (Grotendorst et al., 1981), mitosis (Rutherford and Ross, 1976) and collagen biosynthesis (Eichner et al., 1976; Burke and Ross, 1977). Experimental evidence suggests that PDGF

is specific for mesenchymal cells; it did not affect epidermal cell outgrowth in our in vitro explant model (see Results) nor does it stimulate an epithelial cell line (Westermark and Wasteson, 1983). Heldin et al. (1981) found that receptors for PDGF are restricted to connective tissue and glial cells. However, there is a conflicting report by Aso et al. (1980) that partially purified PDGF added to plasma-supplemented medium results in an increased size and number of epidermal cell colonies compared with control cultures grown in medium with platelet-poor plasma. Recently it was reported that PDGF is very similar or identical to the product of the simian sarcoma virus v-sis oncogene (Waterfield et al., 1983; Doolittle et al., 1983). This was the first demonstrated association between the product of an oncogene and a protein which has a known physiological function in normal cells, although since then other such associations have been discovered (Downward et al., 1984).

Other Blood-Derived Factors

Although Platelet-derived Growth Factor (PDGF) is the name given to the first growth factor purified from platelets, it represents only one of a growing list of growth factors that are associated with platelets. Several growth factors other than PDGF have been found in platelets. Transforming Growth Factor-beta (TGF-beta), first isolated from a neoplastic cell line, has since been found in many normal cells and tissues (see Roberts et al., 1983 for review), including platelets which are a major storage site (Assoian et al., 1983). Purified TGF-beta was shown to enhance connective tissue accumulation in Schilling-Hunt wound chambers embedded under rat skin (Sporn et al., 1983).

Other platelet-associated growth factors are platelet Basic Protein (PBP) and Connective Tissue Activating Peptide III (CTAP III) (Westermark and Wasteson, 1983), which like PDGF, are cationic, but they are smaller than PDGF; PBP is 14-17 K (Paul et al., 1980) and CTAP III is about 9.3 K (Assoian et al., 1983). Other platelet factors reported in the literature (Westermark and Wasteson, 1983) are a 40 K acidic protein that stimulates glial cells (Heldin et al., 1977), a 30-50 K protein that targets a number of cultured cell lines (including a tumor cell line of epithelial origin, Eastment and Sirbasku, 1980) and a 72 K protein, with an isoelectric point between 7.8 and 8.3, that stimulates SV 40 transformed cells (Kepner and Lipton, 1981). None of these has been tested for effects on epidermal cells.

There are also a few growth factors originally isolated from other sources which recent evidence has shown may be associated with platelets. These are Epidermal Growth Factor (EGF) (Cohen, 1962) and Serum Spreading Factor (SSF) (Barnes et al., 1983). EGF, first isolated from mouse submaxillary gland and later identified in human urine, has been shown to stimulate epidermal cell outgrowth in explant cultures of neonatal skin (Cohen, 1962). It has recently been reported that EGF enhances re-epithelialization in partial-thickness wounds in pigs (Brown et al., 1984); and that slow release of EGF in subcutaneous sponge implants promotes dermal healing events in rats (Buckley et al., 1985). EGF is a small monomeric protein (6 K), is extremely stable (Holladay et al., 1976) and has been extensively characterized (Savage and Cohen, 1972; Savage et al., 1973). A variant of EGF has been reported to be associated with serum and platelets (Oka and Orth, 1983). Serum Spreading Factor (SSF) is the name given to two purified components

(65 K and 75 K) that support epithelial cell spreading and movement in culture. SSF was originally derived from serum but more recently it has also been found tightly associated with platelet membranes (Barnes et al., 1983). Other laboratories have isolated closely related components from other sources, which have identical biological activity and which show cross-reactivity in antibody studies. The names given to these components are Epibolin purified from human plasma by Stenn (1981) and Vitronectin purified from human plasma by Hayman et al. (1982).

The fact that several different growth factors are associated with blood platelets suggests that the most effective wound therapy may be the use of a combination of two or more factors that coordinately stimulate several different wound healing events. This idea is supported by the published work of others. Taylor et al. (1983) reported that two serum factors are required for maximal epidermal outgrowth from skin explants. And recently, Assoian et al. (1984) showed that a combination of three purified growth factors from platelets, PDGF, TGF-beta and EGF-variant work coordinately to affect "transformed" growth (anchorage-independent growth) of cells in culture.

The studies reported here show that a platelet homogenate fraction (PHF) added to serum-free medium supports epidermal cell outgrowth in explant cultures (Hebda et al., 1986a) and that PHF can stimulate wound re-epithelialization in animals. This is the first evidence to the investigators' knowledge for support and stimulation of epidermal cell growth by platelets in the absence of other serum or plasma components, and may indicate a "new" platelet-derived factor.

Choice of Experimental Systems

Epidermal cell outgrowth from explants (Hammar and Halprin, 1981)

is a good in vitro model for re-epithelialization because the tissue structure of the epidermis and dermis is partially retained. Although systemic responses are absent in this system, localized interactions, including fibroblast-epidermal, substratum-epidermal, and basement membrane-epidermal interactions remain.

The porcine wound healing model to be used was developed by Eaglstein and Mertz (1978). This method is a modification of the porcine epidermal wound healing model of Winter (1972). In Winter's model, partial thickness wounds are made on the backs of pigs then daily biopsy specimens are examined for re-epithelialization by serial cross-section. The investigators' model permits macroscopic evaluation of epidermal healing. It agrees with Winter's model but is facile enough to permit many precise comparisons between topical treatments. It was subsequently expanded to include an assay of dermal collagen content and biosynthesis according to Hebda et al. (1986b) as a measure of dermal healing. Many different potential methods of improving wound management have been evaluated with this model (Eaglstein and Mertz, 1978; Mertz et al., 1986).

Porcine skin is very similar to human skin, with respect to relative thicknesses of the epidermis and dermis, relative sparsity of hair and the presence of a layer of subcutaneous fat. The large size of the animal (11-15 kg) permits each animal to serve as its own control since many small wounds can be made on each individual and divided into several treatment groups. Other animal models for wound healing have been reported in the literature. Most of these use rodents such as guinea pigs (Rovee et al., 1967), rats (Cohen et al., 1979; Viljanto, 1964), hampsters (Leitzel et al., 1982), and rabbits (Hunt et al.,

1967). Evaluation of wound healing is made by a variety of measurements including collagen deposition in sponge implants (Viljanto, 1964), histology of serial cross-sections (Franklin and Lynch, 1979), visual assessment (Leitzel et al., 1982; Hunt et al., 1967; Rovee et al., 1972) and measurement of tensile strength (Viljanto, 1964; Rovee et al., 1967). All of these models have inherent problems and limitations. The structural features of rodent skin are quite different from human skin and the small size of the animal limits the number of wounds which can be made on each animal. In addition, only one aspect of healing is evaluated--either epidermal resurfacing or dermal collagen production.

The experimental design involves a heterologous system in that the factors are derived from human blood and the target tissue is porcine skin. The investigators chose the design based on most readily available materials and because there is no evidence that excludes cross-reactivity between the two species; blood-derived growth factors thus far purified show little species specificity (Westermark and Wasteson, 1983).

Platelet Factors and Wound Healing

Platelet factors (intracellular or cell membrane-bound) are excellent candidates for wound hormones, because platelets are released at the wound site when bleeding occurs. The research discussed in this report will provide important information about the physiological role of platelets in the healing process. It may lead to the identification of a mechanism for the coordinated regulation of epidermal and dermal healing. It may also lead to the development of therapies utilizing exogenous platelet fractions to augment endogenous factors. These results have 1) provided additional evidence for the pivotal role in

wound healing ascribed to platelets by demonstrating their effect on epidermal cells, 2) shown the effects of several purified growth factors on the epidermis and 3) offered a possible approach to improve the treatment of burn wounds and some other slow healing or chronic wounds (such as venous ulcers).

It is also of great interest to learn how platelet-derived factors function in the overall processes of development, growth, regeneration, repair and aging. This study, which focused on repair and regeneration as they occur in healing skin, may provide essential basic information for future investigations of other events. Epidermal regeneration and dermal repair may be compared and contrasted with developmental growth to understand why wound healing is an imperfect restorative process. The knowledge gained about the basic biochemistry of the skin may ultimately lead to the development of methods for inducing complete tissue regeneration such as the regrowth of a digit or limb.

APPROACH

The following two methods were used to test for a stimulation of epidermal wound healing by platelets--an in vitro skin explant culture study (Hammar and Halprin, 1981) and an in vivo wound healing study using an animal model (Eaglstein and Mertz 1978).

Materials

RPMI 1640 Medium[1], fetal bovine serum (FBS) and antibiotic/antimycotic (AB/AM penicillin 200 units/ml, fungizone 500 ng/ml, streptomycin 200 ug/ml) were obtained from Gibco Laboratories (Grand Island, New York). Chemicals and biochemicals were reagent grade. Human platelet-derived TGF-beta and mouse EGF were generously provided by Drs. Anita Roberts and Michael Sporn (National Cancer Institute). Commercial PDGF was purchased from Bethesda Research Labs (Bethesda, MD). HPLC-purified human PDGF was purchased from Collaborative Research (Lexington, Massachusetts). Domestic Yorkshire swine were supplied by Dalessio's Stock Farm (Plumville, Pennsylvania) and Sullivan Farm (Industry, Pennsylvania). Animals had been raised in confined housing to protect the skin from abrasion and were maintained two weeks prior to use in the University's Central Animal Facility (approved by the American Association for the Accreditation of Laboratory Animal Care). Here they received water and a complete swine grower diet ad libitum and were housed in pens controlled for temperature (20-23°C) and light (12h/12h, light:dark). Human platelets were obtained from the Central Blood Bank (Pittsburgh, PA). The platelets were less than one week old and were physiologically normal.

Platelet Homogenate Fraction (PHF). Concentrated human platelets from the Blood Bank (approximately 10 x from whole blood) were

centrifuged at 400 x g for 10 min to sediment residual red blood cells. The supernatant platelet-rich plasma was centrifuged at 1500 x g for 10 min to yield a platelet pellet and supernatant platelet-poor plasma. The plasma was poured off and discarded and the packed platelets were gently resuspended in an isotonic buffer referred to as platelet incubation medium (100 mM KCl, 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4) (Saliganifcoff et al., 1975). This platelet suspension was centrifuged at 1500 x g for 10 min to yield washed platelets. The platelets were resuspended in a small volume of platelet incubation medium (1 ml/unit of platelets) and homogenized with a Tekmar Tissumizer at 10,000 rpm for 30 sec. The homogenate was centrifuged at 15,000 x g for 10 min to sediment cellular debris which was discarded. The supernatant solution was sterilized by passage through a 0.45 u Millex filter (Millipore Corporation). This Platelet Homogenate Fraction (PHF) was assayed for protein concentration with the Bio-Rad rapid protein assay (Bradford, 1976).

Heat Inactivation of Platelet Homogenate Fraction. PHF was added to serum-free medium at a concentration of 260 ug protein/ml. The suspension was incubated in a boiling water bath (100°C) for 2 min or 5 min then rapidly cooled in an ice bath. After filtration through a 0.45 u Millex filter, the heat-inactivated PHF concentrate was added to medium with or without FBS to produce a ten-fold dilution of PHF (approximately 26 ug protein/ml, some denatured protein being lost during filtration).

Methods

A. In Vitro Studies

Skin Explant Culture Model. For each experiment, a skin specimen was excised from the paravertebral region of a young Yorkshire swine (11-15 kg). The animal, which had received no previous anesthesia or treatment, was anesthetized with ketamine (300 mg, i.m.) and methoxyfluorane (3%, open mask). The skin on the back was prepared for excision as follows: Hair was removed with fine barber's clippers and a razor. The skin was washed sequentially with neutral soap and water, 10% povidone-iodine (1x), 70% methanol (2x) and sterile distilled water (3x). By means of aseptic techniques, 50 x 22 mm skin sheets were excised with a Castro-Viejo electro-keratome set to cut at a depth of 0.2 mm. The sheets were placed in RPMI 1640 medium containing AB/AM and kept at 4°C prior to dissection. The explants were prepared within 24 hr after surgical excision, according to the methods originally developed by Halprin et al. (1979) and adapted to culture of young adult porcine skin by Hammar and Halprin (1981). The skin sheets were kept moist with medium throughout the dissection procedure. Each sheet was examined macroscopically and selected for uniform thickness. Individual explants (1-2 mm squares) were prepared with a sharp scalpel taking care to cut clean edges perpendicular to the surface. Explants were placed dermal-side down in 35 mm plastic culture dishes, four explants/dish. A minimum of ten dishes/group were prepared for each experiment. After the explants air-dried for 15-30 min to obtain adherence to the plastic culture dish, 2 ml of culture medium containing the appropriate test material and AB/AM was added to each dish. The explant cultures were incubated at 37°C with 5% CO_2.

Evaluation of explant outgrowth. Cultures were examined daily for initiation of epidermal cell outgrowth. Explant viability was measured as the number of explants showing outgrowth on Day 3 divided by the total number of explants prepared. This fraction was multiplied by 100 and expressed as the percentage of viable explants (% viability). Once outgrowth was observed, the extent of outgrowth was determined by measuring the longest radius on each growing side of each explant using a phase contrast microscope (100x) fitted with a micrometer eyepiece. The longest radius for each side was measured again on the days indicated in each experiment. The change in the radius for each side was calculated and the maximum difference in the radius between two days of measurement was used as the value for the rate of outgrowth for each explant. Mean values and standard errors of the means (SEMs) were then calculated for each treatment group. Analysis of variance with repeated measures (Sokal and Rohlf, 1981) was applied to the data and appropriate tests were used to determine significant differences in rates of outgrowth between treatment groups. A difference was considered significant when the probability value p was less than or equal to 0.05.

B. In Vivo Studies

Animal Wound Healing Model. Because we wanted to study both the dermis and the epidermis simultaneously during wound healing, we chose to study partial thickness wounds. A partial thickness wound is characterized by disruption of epidermal continuity, loss of all the surface epidermis and destruction of the papillary and superficial reticular dermis. Much of the follicular, glandular and ductile epithelial cells within the dermis are not destroyed. These epithelial cells and ones from the wound edge migrate and resurface the wound.

Young, white Yorkshire pigs 20-30 lbs and approximately two to three months old were used for this study. The pigs were housed individually in our animal facility with controlled temperature (19-21°C) and light (12/12 LD) and were fed a basal diet ad libitum. Although complete environmental studies on wound healing in pigs have not been done, these conditions have yielded reproducible results in our model.

The pigs were clipped with standard animal clippers, and the skin on the entire back and sides was prepared for wounding by washing with mild soap and water and rinsing with water. Other antiseptics were not used because of the potential effect on the healing process. For these experiments the pigs were anesthetized (as described above) and approximately 120-150 rectanglar 7 x 10 mm wounds, 0.3 mm deep were made in the paravertebral and thoracic areas with a Castro-Viejo dermatome. The wounds on each animal were divided into five treatment groups and treated with various extracts from platelets or with purified growth factors as indicated in each experiment. Some wounds were sampled by daily excision for the next seven days. Using the Castro-Viejo dermatome, skin samples were taken, 22 x 33 mm and 0.5 mm deep, that included the entire wound and some surrounding normal tissue. These specimens were incubated in 0.5 M sodium bromide at 37°C for 18 h to separate the dermis and epidermis. The epidermal specimens were mounted on cardboard for evaluation and the dermal specimens were placed in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and stored at -20°C until they were assayed for collagen biosynthetic capacity.

Epidermal specimens were evaluated for healing by the method described by Eaglstein and Mertz (1978). After the dermis and epidermis

were incubated with sodium bromide, the two layers were easily separated with forceps. The moist epidermis was placed on a microscope slide with the stratum corneum (and protruding hair shafts) against the glass. The slide and specimen were pressed against cardboard and the slide carefully removed to leave the epidermis on the cardboard. The specimen was examined for any defect (hole) in the area of the wound. If a crust was present (Days 3-5) this was gently removed with forceps to observe the underlying newly formed epidermis. If a hole was present, the wound was counted as not healed. If no defect was found, the wound was counted as healed. An HT_{50} value (time at which 50% of the wounds were healed) was calculated for each experimental group. The HT_{50} values obtained with the various treatments were compared with vehicle-treated group and untreated control group values to determine whether epidermal healing had been enhanced.

Dermal specimens were evaluated for their capacity to synthesize collagen using a method described by Diegelmann et al. (1975) adapted for analysis of healing wounds by Hebda et al. (1986b). The assay was performed as follows: The dermal wound bed and 1-2 mm of surrounding dermal tissue was cut from the excised dermal specimen with a sharp scalpel. Two to three specimens were combined for each analysis. The tissue was minced on a sterile plastic surface using two sharp scalpels in a rapid opposing motion. The minced tissue was incubated in 4 ml Krebs-Ringer solution without phosphate (Umbreit at al., 1957) containing [^{14}C]-proline (2 uCi/sample) at 37°C for 18 h. After incubation, protease inhibitors (N-ethylmaleimide, phenylmethyl sulfonylfluoride, and ethylenediamine tetracetic acid) were added and the reaction was stopped by freezing. Each sample was homogenized and

dialyzed to remove unincorporated label. Then it was hydrolyzed in 6N HCl under atmospheric N_2 at 100°C for 24 h and lyophilized. The hydrolysate was neutralized, dansylated and chromatographed on a thin-layer chromatography sheet in a solvent system which separates dansyl-proline and dansyl-hydroxyproline. Radioactive areas were cut out and counted in a liquid scintillation counter and the relative collagen biosynthesis (%RCB) was calculated using Peterkofsky's equation (Peterkofsky, 1972).

RESULTS

A. In Vitro Studies

1. Effect of Platelet Homogenate Fraction on Explant Outgrowth

The explants were cultured in medium containing varying concentrations of Platelet Homogenate Fraction (PHF) and fetal bovine serum (FBS). A total of 30 culture dishes were prepared for each of the 8 treatment groups. There were 4 explants per culture dish yielding a total of 120 explants per group. Initiation of outgrowth was observed on Day 3. The measurements of epidermal cell outgrowth on Days 3, 5 and 7 are shown in Table 1. Except for Group 1 (negative control) each group produced 26-69% viability, that is, showed outgrowth. Since the nongrowing (nonviable) explants were evenly distributed throughout each group rather than clustered together in the same dish or dishes, it is unlikely that a loss of viability was due to a culture dish effect. Viability in Groups 2 to 4 (PHF alone) ranged between 45% and 69%, none of which was significantly different from Group 5, the positive control of 5% FBS (67%). However, viability was significantly lower ($p < 0.001$) for Groups 6 to 8 grown with PHF + FBS, 26-37%. Only 2% (two explants) showed outgrowth in medium without PHF or FBS (negative control group) and their outgrowth stopped after Day 5.

The extent of explant outgrowth is plotted in Figure 1 and demonstrate that PHF evoked total outgrowth very similar to the outgrowth obtained with FBS. The outgrowth with 26 ug/ml PHF was the same as the outgrowth with 5% FBS at every time point. With 260 ug/ml PHF, outgrowth was lower on Days 3 and 5 ($p = 0.05$) compared to the other concentrations of PHF. By repeated measures analysis of variance, it was shown that there was a dose-dependent PHF effect. There were

interactions between treatment and time that were not unexpected on Day 7 as the rate of outgrowth decreased during later stages of explant growth with the FBS controls as well. The extent of outgrowth from explants grown with PHF + FBS was the same for all three PHF concentrations and was much less than any concentration of PHF alone in both the extent of outgrowth (radius of the epidermal sheet) and the rate of outgrowth ($p = 0.001$).

In Figure 2, the rates of outgrowth between Days 3 and 7 are shown for different concentrations of PHF either with or without FBS. There was a concentration-dependent increase in the rate of outgrowth with PHF between 26 and 130 ug protein/ml which reached a plateau between 130 and 260 ug/ml. The rates of outgrowth were lower with PHF + FBS than with FBS or PHF alone. This inhibition was not PHF concentration-dependent within the range tested (26-260 ug/ml).

Cultures were examined under a dark field and phase contrast microscope and were found to have structural features characteristic of epidermal sheets from explants (Hammar and Halprin, 1981; Flaxman and Harper, 1975). The cells in the epidermal sheets were typical of keratinocytes based on their morphology and growth kinetics. Cultures grown with PHF (Figures 3A and 3B) had epidermal sheets similar to FBS controls--the sheet was multilayered near the explant, tapering to a single cell layer at the growing edge with many "stretched" epidermal cells (Halprin *et al.*, 1979). Cultures grown with PHF + FBS (Figures 3C and 3D) had abnormal features compared with FBS controls. The epidermal sheet was thick and multilayered throughout, there was no tapering toward a monolayer as is normally found. The cells at the leading edge were not "stretched" or spread but were rounded and piled up. There was

no morphological evidence of cell death. Cells remained adherent to each other and the epidermal sheet remained intact throughout the 7 to 10-day period of observation.

2. Dose-Dependence of Explant Viability and Extent of Outgrowth

Serial dilutions of PHF were made and tested with explant cultures to determine the lower limits of the PHF effect on explant viability and outgrowth. The results shown in Table 2. indicate that PHF supported explant viability at a concentration of 2.6 ug/ml and above. The rate of outgrowth achieved with PHF reached the rate of the FBS control at a PHF concentration between 26 and 130 ug/ml.

3. Effect of Heat on Platelet Homogenate Fraction

Explants were cultured in media containing heat-treated PHF and outgrowth was evaluated and compared with PHF-treated and FBS-treated groups. The results, shown in Table 3., indicate that the stimulatory activity was stable to heat at 100°C for 2 min, but was destroyed by heating at 100°C for 5 min. During the course of these studies, it was found that the inhibitory effect of PHF + FBS was not obtained with every PHF preparation. This is demonstrated in Table 3., which shows that in this experiment PHF + FBS was not inhibitory to viability or outgrowth; whereas, inhibition was found in two previous experiments.

4. Fractionation of Platelet Homogenate Fraction by Molecular Size

PHF was separated into two fractions based on filtration through a membrane that excluded molecules larger than 30 K. The fractions were tested for their effects on explant outgrowth at a concentration equivalent to 26 ug/ml PHF original and the results are shown in Table 4. The "high molecular weight" fraction (>30 K) supported explant viability and outgrowth comparable to the PHF original and thus

contained an epidermal Viability Factor. The "low molecular weight" fraction (< 30 K) did not support viability in serum-free medium as well as PHF original, and the explants that were viable showed a slower rate of outgrowth. However, when the "low molecular weight" fraction was added with 1% FBS, it greatly stimulated the rate of explant outgrowth compared with 1% FBS alone (Figure 4) and thus contained an Epidermal Stimulatory Factor. The stimulatory effect was observed in the later phase of outgrowth (Days 4-6) suggesting that this factor influenced later events, such as mitosis and differentiation (maturation).

5. Heat Inactivation of the Two PHF Fractions

The high and low molecular weight fractions from ultrafiltration were heated in a boiling water bath (100°C) for 5 minutes then rapidly cooled on ice. Activity was measured before and after this heat treatment to determine viability and extent of epidermal outgrowth. The results are shown in Table 5 and Figures 5a and 5b. In Table 5 the results indicate that after heat inactivation, the PHF High MW fraction, which contained the epidermal Viability Factor, still retained activity as reflected by the percentage of viable explants on Day 1 (18%) and the final percentage on Day 3 (38%), which were comparable to viability of PHF High MW without heating. There may have been a partial loss of activity during heating that resulted in a slightly lower percentage of viable explants on Day 2 (18% versus 28% for the PHF High MW without heating). Figures 5a and 5b show the results of heat inactivation of the Stimulatory Factor. The PHF Low MW fraction was added with 1% FBS and the results are shown in Figure 5a. Heat inactivation of PHF Low MW produced a much lower rate of outgrowth on Days 2-4, suggesting inactivation of the Stimulatory Factor. The PHF High MW fraction was

evaluated without fetal bovine serum (FBS) and the results are shown in Figure 5b. Heat inactivated PHF High MW showed a lower rate of outgrowth on Day 3, suggesting the presence of a heat labile Stimulatory Factor. (This Stimulatory Factor may be the same one found in PHF Low MW, which was partitioned between the two fractions.) These results suggest that the Viability Factor in PHF High MW was stable to heating at 100°C, but that the Stimulatory Factor(s) in PHF Low MW (and in PHF High MW) were labile under these conditions. These characteristics distinguish these factors from other known factors of similar molecular weights that have comparable effects on epidermal cells, suggesting that they are previously unknown factors in platelets.

6. Fractionation of Platelet Homogenate Fraction (Preliminary Studies)

Gel filtration of PHF by conventional liquid chromatography and Fast Protein Liquid Chromatography (FPLC) demonstrated that most of the proteins in PHF are larger than 30 K, as previously determined. Figures 6 and 7 show the elution profiles of PHF with each of these chromatographic procedures. It may be seen that FPLC resulted in improved separation of the platelet components.

7. Investigation of Methods for Storing PHF to Preserve Its Activity

We tested several methods for storage of PHF such that the activity with epidermal cells was retained. After storage the PHF preparations were evaluated for activity with explant cultures. The results with respect to explant viability are summarized in Table 6. PHF completely lost activity by slow freezing at -20°C, but activity was retained when freshly prepared PHF was quick frozen in liquid nitrogen (-200°C), lyophilized and stored at -20°C for up to several weeks. The results with respect to the rate of epidermal cell outgrowth are shown in

Figure 8. There was no loss of activity with slow freezing at -20°C but a loss of activity with quick freezing and lyophilization. The PHF preparation also retained activity during short storage (several days) in an ice bucket (0°C) (data not shown). The best conditions for storage of PHF was to quick freeze (-200°C) and lyophilize and store at -20°C or colder, since both activities were retained. (Loss of activity with time and storage has been one of the biggest difficulties in purification of the factors from PHF).

8. Effect of Platelet-Derived Growth Factor on Explant Outgrowth

Explants were cultured in serum-free medium containing commercial partially-purified PDGF, at concentrations of 0.2, 1.0 and 5.0 units/ml. The results are summarized in Table 7. PDGF did not support epidermal cell outgrowth in the absence of FBS. In the presence of 5% FBS, PDGF produced a slight stimulation of epidermal cell outgrowth at 0.2, 1.0 and 5.0 units/ml ($p < 0.05$).

9. TGF-beta and EGF

Initiation of Outgrowth and Viability

Without additional growth supplements, explants in 1% FBS required up to three days in culture to initiate outgrowth (See Figure 9a, panel A). By Day 3, 70% of the samples had migrating epidermal sheets. With the addition of TGF-beta, there was a marked increase in the number of explants growing on Days 1 and 2 as shown in Figure 9a (panels B-E). This increase was observed with all concentrations of TGF-beta tested, 0.01-10 ng/ml. By the third day of culture, the final percentages of growing explants were the same in the untreated control, 70%, and the TGF-beta groups, 65-80%, showing that the controls reached the same level of viability.

In the same experiments, the combined effects of EGF (5 ng/ml) and TGF-beta were evaluated (Figure 9b). In Figure 9b, the first group on the left (panel A) shows the effect of EGF alone. Compared with the control group in Figure 1a panel A, EGF had no effect on Day 1, but produced an increase in the number of growers on Day 2, and by Day 3 90% of the explants were growing indicating a higher explant viability with EGF compared with 70% for the control. The results with TGF-beta plus EGF were comparable to the corresponding results with TGF-beta alone (Figure 9a), showing an increase in the percentage of growing explants on Day 1. But in the presence of 5 ng/ml EGF, this increase was concentration-dependent for TGF-beta suggesting a possible synergistic activity. This synergism was most clearly demonstrated with 10 ng/ml TGF-beta (Figure 9b, panel E--compare with Figure 9a, panel E). Explant viability, as reflected by the number of explants growing on Day 3, was not altered by the addition of TGF-beta (Figure 9b, compare panels B-E with panel A), which is consistent with the results in Figure 9a.

These results show that 1) TGF-beta consistently produced an earlier initiation of outgrowth, but did not change the number of samples that ultimately grew (viability); 2) EGF in medium with 1% FBS increased explant viability to approximately 90%; and 3) EGF alone did not produce early initiation, but 5 ng/ml EGF was synergistic with TGF-beta at 10 ng/ml in producing earlier initiation of outgrowth.

Extent of Epidermal Outgrowth

The early stage of explant cultures is the "migratory phase" (Days 1-3) during which there is no measurable cell division, and the later stage is the "mitotic phase" (Days 4-7) in which there is a large increase in the mitotic index (Hammar and Halprin, 1981). To determine

the effects of TGF-beta and EGF on each phase of outgrowth, viable (growing) cultures were measured daily for four days, then on Day 7. The results are shown in Figures 10a and 10b. In Figure 10a the data show that 1 ng/ml TGF-beta increased the rate of outgrowth during the "migratory phase" (Days 1-3), but had no additional effect on the "mitotic phase" as reflected by the outgrowth from Days 4-7. A concentration of 1.0 ng/ml of TGF-beta was optimal; higher and lower concentrations of TGF-beta (10, 0.1 and 0.01 ng/ml) produced outgrowth that was not significantly different from the control (data not shown).

In Figure 10b the data show that in the presence of EGF, explant cultures grew at the same rate as the controls during the "migratory phase", but had an increased rate of outgrowth during the "mitotic phase". The combination of TGF-beta and EGF produced greater outgrowth during the "migratory phase" (Days 1-3) compared with EGF alone, but there was no additional effect during the "mitotic phase" (Days 4-7) compared with EGF alone. The stimulatory effect on the migratory phase was maximal with 10 ng/ml TGF-beta (Figure 10b), but was also obtained with 1.0 ng/ml TGF-beta (data not shown).

Combined Effects of TGF-beta, EGF, and PDGF on Viability and Outgrowth

Viability and extent of epidermal outgrowth were measured with PDGF alone and with PDGF plus 5 ng/ml EGF and 1 ng/ml TGF-beta. The results are shown in Table 8. PDGF-treated groups did not grow better than the controls with respect to either viability or extent of outgrowth, and in fact there was lower viability and less outgrowth on Day 1 in the presence of PDGF.

11. The Effect of Metabolic Inhibitors on the Outgrowth-promoting Activity of PHF, TGF-beta and EGF

a. Cycloheximide is a potent inhibitor of de novo protein synthesis. We tested the effects of adding 25 ug/ml cycloheximide to explant cultures in medium containing various supplements and growth factors. The results in Table 9 show that cycloheximide completely blocked epidermal outgrowth from explants with every supplement tested. Therefore, we conclude that de novo protein synthesis is essential for the initiation of outgrowth.

b. Monensin is an inhibitor of cellular secretion. We tested the effects of adding 10^{-6} M monensin to explant cultures in medium containing various supplements and growth factors. The results in Table 10 indicate that monensin did not block the initiation of outgrowth with FBS, PHF or TGF-beta but that there was a slight decrease in the rate of outgrowth on Day 7. Therefore, under these conditions, cells in the explants do not secrete factors that are essential for the initiation of outgrowth, i.e. attachment, spreading and migration; but, the cells may secrete factors that enhance the rate of outgrowth. This result is especially interesting with respect to TGF-beta, which we have shown to produce early initiation of outgrowth. This experiment suggests that TGF-beta acted directly to produce this effect (possibly by inducing membrane receptor expression) rather than by causing the secretion of an autocrine or paracrine attachment or spreading factor.

B. In Vivo Studies

1. Platelet Preparations

These preliminary studies were conducted to determine the most

effective concentration of platelets and frequency of applications. The platelet preparations described below were applied topically to wounds according to a regimen outlined in Table 11.

a. Fresh platelet suspensions. Recently outdated human platelets (<1 week old) were obtained from the blood bank and collected by centrifugation (100 x g for 15 min). When necessary, red blood cells were first removed by low speed centrifugation (400 x g for 10 min). The platelets were washed once with phosphate-buffered saline and suspended in phosphate-buffered saline for application. Two separate experiments were conducted using a total of four animals. Two or three wounds per group were sampled each day for 5 days. Collagen biosynthesis was measured in trypsin-split dermal samples. No difference was found in percent RCB (relative collagen biosynthesis) between platelet-treated, vehicle-treated or untreated groups. We interpret this to indicate that platelets were not releasing their growth factors at the site of injury.

b. Thrombin-activated platelets. Thrombin (10 units/ml) was added to fresh platelet suspensions, causing the platelets to aggregate (and release the contents of their alpha-granules). The suspensions were then applied to wounds as before. Two or three wounds per group per day were sampled by excision. Both experimental animals exhibited abnormally long bleeding times on Days 2-5. We attribute this to a systemic depletion of serum clotting factors by the thrombin, since we have never observed such a response before or since. Therefore, as used here, thrombin was found to be an unacceptable activator of platelets.

c. Platelet homogenate fraction. We homogenized fresh platelets with a tissuemizer and applied the homogenates to wounds as previously described. Samples were taken for analysis of epidermal healing and dermal collagen biosynthesis (five wounds per group per day). The dermal samples were lost due to a power failure which interupted refrigeration. Epidermal samples were evaluated and we found a slight decrease in healing time (HT_{50}) for group I (3×10^{10} pl/ml) compared with group IV (vehicle). The results are shown in Table 12.

2. Topical Application of Platelet Homogenate Fraction (PHF).

Four pigs were used in this experiment. Treatments (PHF or vehicle) were applied immediately after wounding and each subsequent day. Approximately 50 ul/wound was applied with a plastic pipette to cover the wound bed and incubated in place for 5 min. Five wounds from each group were sampled by excision each day for Days 2-7 after wounding and evaluated for epidermal resurfacing and 2 wounds/day for Days 1-7 were analyzed for collagen biosynthesis. The results for epidermal resurfacing are summarized in Table 13. The results of dermal collagen biosynthesis are shown in Table 14. This experiment indicates that there is no increase in the rate epidermal healing and no increase in dermal collagen biosynthesis with daily 5 min topical application of PHF. One of the possible explanations for the absence of a healing effect is that the method of applying platelet homogenate was not effective. Therefore, the following experiment was designed to improve the method of exposing wounds to platelet homogenate.

3. Topical Application of PHF with Occlusion.

Two pilot studies were conducted using two animals for each study.

In the first pilot the wounds were treated with 50 ul PHF (10 mg protein/ml) or vehicle and immediately covered with an adhesive film dressing. The dressing was kept in place for 6 hr then removed after which time the wounds were air-exposed. Sample wounds (5/group/day) were excised and evaluated as before. Control untreated wounds, as previously described, were also evaluated. The results for this evaluation of epidermal resurfacing are shown in Table 15 A. Since only two animals were used, complete data analysis was not conducted. However, the trends for epidermal healing indicate that healing was accelerated in the two treated groups (active and vehicle) compared with control untreated wounds. However, there was no difference in healing between PHF- and vehicle-treated wounds.

In a second pilot study, the protocol was repeated with application of PHF followed by 2 hr of occlusion. The results for epidermal resurfacing are shown in Table 15 B. The results of epidermal resurfacing are similar to those obtained with 6 hr of occlusion. PHF- and vehicle-treated wounds did not differ and both healed slightly faster than control untreated wounds.

4. Measurement of Collagen Synthesis in Normal Nonwounded Porcine Skin after Repeated Sampling.

Daily sampling of wounds involves excising each wound, and therefore, making another larger wound. In order to determine the effect of repeated sampling from the same animal on the skin specimens with respect to collagen synthesis, we measured the Relative Collagen Biosynthesis (%RCB) in normal nonwounded skin samples taken from the back of an animal for five consecutive days. The results, shown in Figure 11, indicate a slight variation in the measurements with no large

peaks or troughs. This small degree of variation should not interfere with results obtained in wounds.

5. Time Course for Incorporation of ^{14}C-Proline into Protein by Minced Porcine Skin.

A control study was conducted to determine optimal incubation time for the collagen biosynthesis assay. A sample of normal nonwounded pig skin was minced fine and divided into seven equal parts. ^{14}C-Proline was added to each sample and incubated at 37°C for various times up to 24 hr. The assay was then conducted as usual (see Hebda et al., 1986), and the samples were evaluated for total incorporation of ^{14}C-Proline into nondialyzable material and for Relative Collagen Biosynthesis (%RCB). The results are shown in Figures 12 and 13. Significant incorporation of the label was first observed at 6 hr, reached a plateau and was stable from 10-18 hr and decreased somewhat at 24 hr (Figure 12). The %RCB at these time points varied slightly and the relative maximum %RCB was measured at 18 hr (Figure 13). Therefore, 18 hr was indicated to be the optimal incubation time for pig skin samples.

6. Wound Healing with Platelet Homogenate Fraction (PHF) in Polyethylene Oxide Hydrogel.

PHF was allowed to be absorbed by polyethylene oxide hydrogel (Vigilon^{TM}) which was then placed over wounds. The results indicated that this treatment had no effect on epidermal wound healing, compared to the Vigilon^{TM} alone (data not shown).

7. Wound Healing with PHF in Carboxymethyl Cellulose.

PHF was mixed with a slurry of 2.5% carboxymethyl cellulose in platelet incubation medium. The slurry was applied to wounds and the area was covered with an adhesive film dressing (Tegaderm^{R}). The results of this treatment on epidermal and dermal wound healing are shown in Table 16 and Figure 14. In Table 16, epidermal healing is

expressed as HT_{50} (healing time 50), the time at which 50% of the wounds were re-epithelialized. PHF at 30 ug/wound had a lower HT_{50} (faster healing) than the vehicle alone. However, the vehicle had a higher HT_{50} (slower healing) than the untreated samples. We believe this was due to an experimental artifact, the aggressive adherence of the vehicle and dressing to the wounds, which may have caused rewounding when the dressing was removed. This problem will be remedied in future studies by extensive hydration before removal of the dressing. However, the data for the vehicle and the two concentrations of PHF were obtained from wounds exposed to the same experimental conditions of potential rewounding, and in these three groups, we observed a dose-dependent increase in healing with PHF.

The effects on dermal collagen biosynthesis are shown in Figure 14. PHF at the lower concentration (6 ug/wound) was not different from vehicle alone and PHF at the higher concentration (30 ug/wound) produced a lower %RCB on Days 1 and 5 compared with the vehicle. It is possible that the higher concentration of PHF contained inhibitory factors for dermal collagen biosynthesis.

8. Partial-thickness Wound Healing Studies with Transforming Growth Factor-beta.

TGF-beta was tested for its effect on wounds in animals and the results shown in Table 17 and Figure 15. In Table 17, epidermal healing is expressed at HT_{50} (healing time 50), the time at which 50% of the wounds were re-epithelialized. TGF-beta at 0.25 ug/wound had a slightly lower HT_{50} (faster healing) than the vehicle alone. At a higher concentration of 1.2 ug/wound, TGF-beta had a higher HT_{50} (slower healing) than vehicle alone. Since TGF-beta has been found to be

inhibitory to some cells at higher concentrations, it may be worthwhile to repeat the wound healing trial with lower concentrations of TGF-beta. It may also be useful to test TGF-beta in different vehicles and in combination with other growth factors, such as epidermal growth factor (EGF), which has been found to act synergistically with TGF-beta. At present, these studies cannot be done because purified TGF-beta is not readily available in quantities required for animal wound healing studies.

Figure 15 shows the effects of TGF-beta on dermal collagen biosynthesis. Although the data are incomplete, they suggest that TGF-beta at 1.2 ug/wound produced a higher %RCB compared to the other treatments on Day 3. This finding agrees with the work of others who have shown that TGF-beta stimulates collagen synthesis in animals.

C. Recent Clinical Findings

Throughout these studies, we have been working with clinicians who share our interests in wound healing. Recently, we have had positive results in a study of patients with venous leg ulcers. We treated the venous ulcers of two patients with topical application of platelet fractions prepared from the patients' own platelets. Although this clinical project is outside the scope of the work sponsored by our current Army contract, it is based on our laboratory studies of platelet fractions (PHF) and epidermal explant cultures. The results are highly relevant to the goals of this project.

Two patients with leg ulcers have been studied to date. Each of these patients had a history of chronic cutaneous ulcers for over three years, and both had been unresponsive to topical treatment and skin grafting. Each patient was treated in a vehicle-controlled trial of

topical platelet fractions. During the course of treatment, the wounds were evaluated macroscopically; and when changes were observed, the wounds were biopsied and the specimens were examined histologically. In each case there was a positive effect on epithelialization (epidermal wound healing) and granulation tissue formation (dermal wound healing) in the wounds treated with platelet fractions compared with the vehicle-treated wounds.

The effect on epithelialization was dramatic. In patient #1 (N.F.) a new stratum corneum was observed in the wound receiving the patient's platelet fraction (see Figure 16). Histology showed that there was keratin present as well as new epidermis and hyperplastic squamous epithelium. Following treatment patient #1 continued to do well, with new epidermis on regions of the ulcer previously resistant to epithelialization. However, the patient developed an infection which eventually caused sloughing of the epidermal layer.

In patient #2 (H.B.) two ulcers were treated in a double-blind, vehicle-controlled study. Platelet Homogenate Fraction (PHF) was prepared as described previously. The wounds were covered with occlusive dressing and treated weekly. Improvement was determined by macroscopic and microscopic assessment of healing (see Figures 17a and 17b). After three weeks of therapy, the code was broken and the results showed that--

a. in the PHF-treated wound there was a hypertrophic epidermis in a central band across the wound bed, probably originating from pre-existing viable epidermis (see Figure 17a). (Dermatopathologist's note: Such hyperplastic epidermis has never been observed previously in such an ulcer.)
b. in the vehicle-treated wound there was no change and no evidence of epithelialization. Histology showed areas of necrotic epidermal tissue (see Figure 17b).

These clinical trials will be continued as suitable patients are enrolled in the Cutaneous Ulcer Rehabilitation and Education Program.

These results provide evidence of a positive effect of platelet fractions on epidermal wound healing. In addition, there was evidence that dermal healing (granulation tissue formation) was also enhanced.

DISCUSSION

A. In Vitro Studies

Platelet Homogenate Fraction (PHF)

This study provides evidence that human platelets contain factors that support epidermal cell viability, initiate onset of epidermal outgrowth and stimulate the rate of epidermal outgrowth from porcine skin explant cultures without serum supplement. The properties and activities of PHF were compared and contrasted with known platelet-derived factors and other growth factors. The significance of these findings, especially with respect to wound healing, are discussed below.

An explant was considered viable if it demonstrated any epidermal cell outgrowth by Day 3. The results demonstrate that a factor (or factors) in PHF supported explant viability to approximately the same extent as positive control cultures grown with 5% FBS. The factor in PHF responsible for explant viability is not likely to be PDGF since commercial PDGF alone did not produce viable explant outgrowth (Table 7.). The demonstration by Heldin et al. (1981) that an epithelial cell line lacks cell surface PDGF receptors supports the conclusion that PDGF does not affect epithelial cells directly. These experiments show that epidermal cell migration was initiated in PHF-supplemented medium without serum. Since the initiation of explant outgrowth (epidermal cell migration) requires attachment and spreading, PHF contained factors that permitted or facilitated both of these events. PHF (26 ug/ml) initiated outgrowth as effectively as 5% FBS, whereas, higher concentrations were less effective (See Figure 1.). It may be that the concentration of outgrowth initiation factor in PHF exceeded the effective range and actually inhibited initiation.

PHF stimulated the rate of outgrowth in a concentration-dependent manner (Table 2. and Figure 2.). The remaining question is the identity of the factor responsblie for this effect. It is possible that PDGF may act synergistically with another factor present in PHF (and serum) to stimulate epidermal cell outgrowth. This idea is supported by our finding that PDGF + FBS was stimulatory for epidermal outgrowth (Table 7.). The possibility that PDGF + FBS has an effect on epithelial cells is also suggested by the report of Aso et al. (1980) that thrombin-treated platelets plus plasma increase the size and number of colonies in keratinocyte cultures. However, both our study and that of Aso et al. (1980) used PDGF that may have contained trace amounts of other factors.

In addition to PDGF, other growth factors which have been found in platelets include Platelet Basic Protein (Paul et al., 1980), Connective Tissue Activating Peptide III (Castor et al., 1977), Transforming Growth Factor-beta (Childs et al., 1982), an Epidermal Growth Factor variant (Assoian et al., 1984; Oka and Orth, 1983) and others (Westermark et al., 1983). These factors have been found to affect connective tissue cells but not epithelial cells. Therefore, ours is the first demonstration of an effect of platelet-derived factors on epidermal cell outgrowth. One or several of the factors in PHF which affect connective tissue cells may have influenced epidermal cells indirectly through the dermal element present in the explant cultures. Such indirect effects on the epidermis cannot be ruled out in explant cultures since they contain a portion of the papillary (upper) dermis.

Factors that influence epidermal cells directly have been found in plasma and serum: Epidermal Growth Factor (Savage and Cohen, 1972),

Serum Spreading Factor (Barnes et al., 1980), Epibolin (Stenn, 1981) and Fibronectin (Yamada and Olden, 1978). Since PHF's stimulatory effect was stable at 100°C for 2 min, (Table 3.) it is probably not Fibronectin which is heat labile at 80°C for 2 min. The finding that the activity is destroyed by heating at 100°C for 5 min eliminates Epidermal Growth Factor, Connective Tissue Activating Peptide III and Transforming Growth Factor-beta, but leaves the possibility that Epibolin or Serum Spreading Factor is responsible for the viability effect. The activity of PHF is not distinguishable from Epibolin or Serum Spreading Factor based on heat stability (Table 3.) or size (Table 4.). Epibolin was first identified as the factor that causes epiboly, the spreading and migration of epidermal cells about the dermis in floating explants. Epibolin has been found in plasma and serum in approximately equivalent amounts. It has not been reported to be in platelets but one explanation for our observations is that Epibolin is associated with platelets. Serum Spreading Factor has been purified from serum and has a high degree of homology with and may be related to Epibolin. It is also possible that we are observing a "new" platelet-derived factor which influences epidermal cells.

PHF and FBS together resulted in inhibition of viability, decreased total outgrowth and slower rates of outgrowth (Table 1. and Figures 1. and 2.). FBS contains factors released by platelets and combined with PHF may have inhibited explant outgrowth by producing 1) increased amount of an inhibitor to an effective concentration, 2) excess amounts of stimulatory factors for attachment, spreading or migration that actually inhibit at higher concentrations, 3) inhibitory products by combining PHF and FBS, or 4) an increased amount of a cytotoxin that

kills a portion of the epidermal cell population. The fourth possibility may probably be eliminated because microscopic examination showed no cell death but rather a piling up of cells in the epidermal sheet. The piled up cells did not exhibit a migratory morphology but rather were rounded and poorly attached to the dish (Figure 3.). The possibility of a factor being stimulatory at low concentrations and inhibitory at higher concentrations (#2) is a plausible one since such bifunctionality has been observed with higher concentrations of Serum Spreading Factor (Barnes et al., 1980) and Transforming Growth Factor-beta (Roberts et al., 1985). The inhibitory effect was not observed in every preparation of PHF suggesting that it either was not universally present in platelets or was very labile and lost during processing.

The importance of platelets in the wound healing process has been a subject of active investigation (Seppa et al., 1982; Ross, 1980; Deuel et al., 1982; Knighton et al., 1982). It is known that platelet-derived factors stimulate events crucial to normal dermal wound healing (fibroplasia, collagen biosynthesis and angiogenesis). However, an effect on epidermal wound healing has not been proven, although the possibility has been suggested (Aso et al., 1980). In this study a platelet fraction supported and stimulated epidermal cell outgrowth from skin explants. These results show that platelet factors can influence epidermal cell attachment and migration, either directly or through dermal interactions. Our results support the hypothesis that platelets play an important role in the healing process and extend this role to encompass epidermal, as well as dermal healing.

Purified Growth Factors

In these experiments TGF-beta had a stimulatory effect on epidermal cells in culture, producing an early initiation of outgrowth and an increased rate of outgrowth during the migratory phase of culture. The stimulatory effect of EGF on the rate of epidermal outgrowth, previously demonstreated by others (Fischer et al., 1980), was also observed in these experiments. In addition, EGF produced greater explant viability. The combination of TGF-beta and EGF resulted in early initiation of outgrowth (TGF-beta effect), increased net explant viability (EGF effect) and an increased rate of outgrowth (TGF-beta effect on Days 1-3, EGF effect on Days 4-7). The effect of TGF-beta and EGF together were primarily additive although the effect on initiation of outgrowth shown in Figure 9b, panel E, is suggestive of a synergistic activity. From these studies, we conclude that in this system TGF-beta is an important factor in initiating and stimulating epidermal cell migration, and that EGF acts primarily as a mitogen for epidermal cells (Rheinwald and Green, 1977). The combination of TGF-beta and EGF produced the greatest explant viability and rate of outgrowth.

Moses et al. (1985) reported that TGF-beta inhibits growth (mitosis) in human foreskin keratinocytes as determined by incorporation of tritiated thymidine into cultures of rapidly dividing cells. We have shown a stimulatory effect of TGF-beta on initiation of epidermal cell migration in explants and on the extent of epidermal outgrowth during the migratory phase of explant culture. These observations suggest that TGF-beta may direct epidermal cells toward migration rather than division.

Although PDGF has a stimulatory effect on mesenchymal cells, it was not found to stimulate epidermal cell outgrowth from explants in these studies. Since the PDGF-receptor appears to be specific for mesenchymal cells, this finding is not surprising. Our study additionally demonstrates that HPLC-purified PDGF had no indirect stimulatory effect on epidermal outgrowth via the dermal tissue in the explants when PDGF was added alone or with TGF-beta and EGF. We showed that a partially-purified commercial preparation of PDGF plus FBS had a slight stimulatory effect on epidermal outgrowth (Table 7). This may have been due to the presence of a slight amount of TGF-beta or another platelet-derived factor in the commercial PDGF preparation used.

Roberts and Sporn (1985) have discussed the role of TGF-beta in other culture systems. TGF-beta was named for its ability to produce anchorage-independent growth of cells in soft agar culture. Our studies suggest that TGF-beta also promotes anchorage-dependent outgrowth--attachment, spreading and migration across a plastic substratum.

Takashima and Grinnell (1985) reported that epidermal explants require three to four days to express fibronectin receptor function in vitro and that this receptor function is required for continued outgrowth. One explanation for our observations is that by Day 1 of culture TGF-beta induces the expression of the fibronectin receptor or the receptor of another spreading factor and permits outgrowth to begin.

It has been proposed that TGF-beta is a physiologically important wound hormone. It is stored in platelets and released at the site of injury. TGF-beta stimulates fibrosis when injected into wound chambers implanted in the backs of rats (Sporn et al., 1983) and when injected

into the nape of the neck of the newborn mouse (Roberts and Sporn, 1985). This study enlarges the role of TGF-beta to include the epidermis. By accelerating the start of epithelialization, TGF-beta may be an important initiating signal for epidermal wound healing.

B. In Vivo Studies

Platelet Homogenate Fraction (PHF)

Daily topical treatment with Platelet Homogenate Fraction (PHF) did not affect epidermal resurfacing (Table 13) compared to vehicle-treated or untreated wounds. Nor was dermal healing as measured by collagen biosynthesis stimulated by the application of PHF compared with vehicle alone. Since the period of exposure (5 min) may have been too short to be effective, treatments were next applied in a single dose on Day 0 with occlusion to extend the exposure period.

With 6 hr of occlusion both the active- and vehicle-treated wounds showed enhanced healing compared with untreated (air exposed) wounds. However, there was no difference in healing between the two treatments. Apparently, occlusion alone was responsible for accelerated epidermal healing. Even with 2 hr of occlusion there was accelerated epidermal healing in both treatment groups but no additional enhancement with PHF. Therefore, in these two pilot studies an effect by PHF may have been masked by the occlusion effect. This finding indicates that brief occlusion (2-6 hrs) may be sufficient to promote healing. It has already been established that occluded wounds heal faster than air-exposed wounds (Eaglstein and Mertz, 1978). These data suggest that in order to achieve this beneficial effect, occlusion immediately after wounding may be necessary and that a relatively brief period of occlusion (less than a day) may be sufficient.

In addition, these studies have shown that PHF in a viscous vehicle (carboxymethylcellulose) stimulated epidermal wound healing in an experimental animal model (Table 16). Related clinical studies also demonstrated an enhancement of epithelialization in chronic venous ulcers in humans (Figures 16 and 17). These findings indicate that physiological growth factors applied topically to wounds can produce an accelerated healing response. Further investigation of the factors in PHF that affect epidermal and dermal wound healing is needed to determine 1) which factors are important and 2) what are the optimal conditions for treatment.

CONCLUSIONS

This investigation has shown that platelet-derived components support the growth of epidermal cells *in vitro* in explant cultures, and that PHF stimulates epidermal wound healing in wounds in animals suggesting that platelets may have a function in epidermal healing. Our findings are encouraging and emphasize the need to purify the active factors from platelets to develop a stable formulation that can be used as a safe, effective and practical wound therapy.

RECOMMENDATIONS

1. Platelet Homogenate Fractions should be further fractionated and the explant culture model utilized to identify the active components.
2. A purified active fraction should then be evaluated in vivo using the porcine wound model. A purified fraction may be compounded with a neutral topical vehicle for more efficacious treatment of wounds. Factors that test positive for stimulating epidermal and dermal wound healing events should be tested alone and in combination to determine optimal treatment conditions.
3. A treatment which has been successful in stimulating wound healing in the animal model should be evaluated in the clinic with human volunteers.
4. Factors that test positive in #1 should be tested for beneficial effects in patients with venous ulcers.

These recommendations are addressed in Dr. Hebda's Research Proposal "The Effects of Platelet Factors on Epidermal Resurfacing during the Repair of Partial-thickness Wounds" which is a continuation of this investigation.

REFERENCES

Aso K, Kondo S and Amano M (1980) Platelet-dependent serum factor that stimulates the proliferation of epidermal cells in vitro. In: Current Problems in Dermatology, (Ed. by I.A. Bernstein and S. Makoto), Vol. 10, pp. 83-89. University of Tokyo Press, New York.

Assoian RK, Komoriya A, Meyers CA, Miller DM, Sprorn MB (1983) Transforming growth factor-beta in human platelets. Identificatin of a major storage site, purification, and characterization. J Biol Chem 258:7155-7160.

Assoian RK, Grotendorst GR, Miller DM, Sporn MB (1984) Cellular transformation by coordinated action of three pepetide growth factors from human platelets. Nature 309,804-806, 1984.

Balk SD (1971) Calcium as a regulator of the proliferation of normal, but not of transformed, chicken fibroblasts in a plasma-containing medium. Proc Natl Acad Sci USA 68,271-275.

Barnes D, Wolfe R, Serrero G, McClure D, Sato G (1980) Effects of a serum spreading factor on growth and morphology of cells in serum-free medium. J Supramol Struct 14,47-63.

Barnes DW, Silnutzer J, See C, Shaffer M (1983) Characterization of human serum spreading factor with monoclonal antibody. Proc Natl Acad Sci USA, 80:1362-1366.

Bradford MD (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle to protein-dye binding. Anal Biochem 72:248-254.

Brown GL, Schultz G, Brightwell JR, Tobin GR (1984) Epidermal growth factor enhances epithelialization. Surg Forum 35:565-567.

Buckley A, Davidson JM, Kamerath CD, Wolt TB, Woodward SC (1985) Sustained release of epidermal growth factor accelerates wound repair. Proc Natl Acad Sci USA 82:7340-7344.

Burke JM, Ross R (1977) Collagen synthesis by monkey arterial smooth muscle cells during proliferation and quiescence in culture. Exptl Cell Res 107:387-395.

Castor CW, Ritchie JC, Scott ME, Whitney SL (1977) Connective tissue activation. XI. Stimulation of glycosaminoglycan and DNA formation by a platelet factor. Arthrit Rheum 20,859-868.

Childs CB, Proper JA, Tucker RF, Moses HL (1982) Serum contains a platelet-derived transforming growth factor. Proc Natl Acad Sci USA 79,5312-5316.

Cohen S (1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J Biol Chem 237:1555-1562.

Cohen IK, Clark DM, Diegelmann RF (1979) Onset and localization of collagen synthesis during wound healing in open rat skin wounds. Proc Soc Exp Bio Med 160:458-462.

Deigelmann RF, Rathkopf LC, Cohen IK (1975) Measurement of collagen biosynthesis during wound healing. J Surg Res 19:239.

Deuel TF, Senior RM, Huang JS, Griffin GL (1982) Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. J Clin Invest 69,1046-1049.

Doolittle RF, Hunkapil MW, Hood LG, Devare SG, Robbins KL, Aaronson SA, Antoniades HN (1983) Simian sarcoma virus oncogene, V-sis is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221:275-277.

Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD (1984) Close similarity of epidermal growth factor receptor and v-erbB oncogene protein sequences. Nature (London) 307:521-527.

Eaglstein WH, Mertz PM (1978) New method for assessing epidermal wound healing: The effects of triamcinolone acetonide and polyethylene film occlusion. J Invest Dermatol 71:382-384.

Eastment CT, Sirbasku, DA (1980) Human platelet lysate contains growth factor activities for established cell lines derived from various tissues of several species. In Vitro 16,694-705.

Eichner J, Cardinale G, Fuller G (1976) Effects of platelets on collagen synthesis in cultured rabbit aortic smooth muscle cells. Fed Proc 35:679.

Fischer SM, Viaje A, Mills GD, Slaga TJ (1980) Explant methods for epidermal cell culture. In: Methods in Cell Biology, (Harris cc, Trump BF, Stoner RD, eds) Academic Pressn Vol. 21A, pp. 207-227.

Flaxman BA, Harper RA (1975) In vitro analysis of the control of keratinocyte proliferation in human epidermis by physiologic and pharmacologic agents. J Invest Dermatol 65,52-59.

Fogdestam I, Gottrup F (1980) Biomechanical methods in wound healing research with special reference to skin and gastro-intestinal tract. In: Biology of Collagen (Viidik A, Vuust J, eds.) Academic Press, New York pp. 363-371.

Franklin JD, Lynch JB (1979) Effects of topical applications of epidermal growth factor on wound healing. Plast Recon Surg 64:766-770.

Gould BS (1968) Collagen biosynthesis. In: Treatise on Collagen. (BS Gould, ed) London Academic Press, London, Vol. 2A, pp. 139-188.

Grant ME, Prockop DJ (1972) The biosynthesis of collagen. N Engl J Med 286(4):194-199.

Grotendorst GR, Seppa HE, Kleinman HK, Martin GR (1981) Attachment of smooth muscle cells to collagen and their migration toward platelet-derived growth factor. Proc Natl Acad Sci USA 78,3669-3672.

Halprin KM, Lueder M, Fusenig NE (1979) Growth and differentiation of postembryonic mouse epidermal cells in explant cultures. J Invest Dermatol 72,88-98.

Hammar H, Halprin K (1981) Epidermal cell growth. In: The Epidermis in Disease (Ed. by R. Marks and E. Christophers), MTP Press, pp. 243-265.

Harker LA, Roass R, Slichter SJ, Scott RC (1976) Homocystine-induced arteriosclerosis: The role of endothelial cell injury and platelet response in its genesis. J Clin Invest 58,731-741.

Hayman EG, Engvall E, A'Hearn E, Barnes D, Pierschbacher M, Ruoslahti E (1982) Cell attachment on replicas of SDS polyacrylamide gels reveals two adhesive plasma proteins. J Cell Biol 95:20-23.

Hebda PA, Alstadt SP, Hileman WT, Eaglstein WH (1986a) Support and stimulation of epidermal cell outgrowth from porcine skin explants by platelet factors. British J Dermatol 115,529-541.

Hebda PA, Kao WW-Y, Mertz PM, Eaglstein WH (1986b) Collagen biosynthesis and collagen content as a measure of dermal healing in experimental wounds in domestic swine. In: Swine in Biomedical Research (Tumbleson ME, ed.) Plenum Press, New York; pp 303-311.

Heldin C-H, Westermark B, Wasteson A (1981) Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. Proc Natl Acad Sci USA 78,3664-3668.

Heldin C-H, Wasteson A, Westermark B (1977) Partial purification and characterization of platelet factors stimulating the multiplication of normal human glial cells. Exptl Cell Res 109:429-437.

Hunt TK, Twomey P, Zederfeldt B, Dunphy JE (1967) Respiratory gas tensions and pH in healing wounds. Amer J Surg 14:302-307.

Holladay LA, Savage CR, Cohen S, Puett D (1976) Conformation and unfolding thermodynamics of epidermal growth factor and derivatives. Biochemistry 15:2624-2633.

Kepner N, Lipton A (1981) A mitogenic factor for transformed fibroblasts from human platelets. Cancer Res 41:430-432.

Kohler, N. and Lipton, A. (1974) Platelets as a source of fibroblast growth-promoting activity. Exptl Cell /Res 87,297-301.

Krizek TJ, Robson MC, Wray RC (1968) The functional anatomy of skin. In: The Management of Trauma. (W Ballinger et al. ed) W. B. Saunders Company, Philadelphia, pp. 650-702.

Leitzel K, Cano C, Marks J, Lipton A (1982) Failure of nerve growth factor to enhance wound healing in the hamster. J Neurosci Res 8:413-417.

Mertz PM, Hebda PA, Eaglstein WH (1986) A porcine model for evaluating epidermal wound healing. In: Swine in Biomedical Research (Tumbleson ME, ed.) Plenum Press, New York; pp. 291-302, 1986.

Moses HL, Tucker RF, Leof EB, Coffey Jr RJ, Halper J, Shipley GD (1985) Type-beta transforming growth factor is a growth stimulator and a growth inhibitor. In: Cancer Cells. 3. Growth Factors and Tranformation. (Feramisco J, Ozanne B, Stiles C, eds.) Cold Spring Harbor Laboratories, pp 65-71.

Oka Y, Orth DN (1983) Human plasma epidermal growth factor/beta-urogastrone is associated with blood platelets. J Clin Invest 72,249-259.

Paul D, Niewiarowski S, Varma KG, Rucinski B, Rucker S, Lange E (1980) Human platelet basic protein associated with antiheparin and mitogenic activities: Purification and partial characterization. Proc Natl Acad Sci USA 77,5914-5918.

Pillsbury DM, Shelley WB, Kligman AM (1965) Corium and subcutaneous tissue. In: Dermatology. W. B. Saunder Company, Philadelphia, p. 67.

Pillsbury DM, Shelley WB, Kligman AM (1965) Hair and the glands of the skin. In: Dermatology. W. B. Saunders Company, Philadelphia, p. 40.

Rheinwald JG, Green H (1977) Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature 265:421-424.

Roberts AB, Frolik CA, Anzano MA, Sporn MB (1983) Transforming growth factors from neoplastic tissues. Fed Proc 42:2621-2626.

Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF, Sporn MB (1985) Type beta transforming growth factor: a bifunctional regulator of cellular growth. Proc Natl Acad Sci, USA 82:119-123.

Roberts AB, Sporn MB (1986) Transforming growth factors. Cancer Surveys 4,683-706.

Ross R (1980) Fibroblast proliferation induced by blood cells. Agents Actions [Suppl] 7,81-84.

Ross R, Vogel A (1978) The platelet-derived growth factor: Review. Cell 14,203-210.

Ross R, Glomset J, Kariya B, Harker L (1974) A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc Natl Acad Sci USA 71,1207-1210.

Rovee DT, Kurowsky CA, Labun J (1972) Local wound environment and epidermal healing. Mitotic response. Arch Dermatol 106:330-334.

Rovee DT, Lowell J, Miller CA (1967) Tensiometric analysis of epidermal healing in the plantar skin of quinea pigs. J Invest Dermatol 48:266-267.

Rutherford RB, Ross R (1976) Platelet factors stimulate fibroblasts and smooth muscle cells quiescent in plasma serum to proliferate. J Cell Biol 69:196-203.

Salganicoff L, Hebda PA, Yandrasitz J, Fukami MH (1975) Subcellular fractionation of pig platelets. Biochim Biophys Acta 385,394-411.

Savage CR, Cohen S (1972) Epidermal growth factor and a new derivative. Rapid isolation procedures and biological and chemical characterization. J Biol Chem 247,7609-7611.

Savage CR, Hash JH, Cohen S (1973) Epidermal growth factor. Location of disulfide bonds. J Biol Chem 248:7669-7672.

Seppa H, Grotendorst G, Seppa S, Schiffmann E, Martin GR (1982) Platelet-derived growth factor is chemotactic for fibroblasts. J Cell Biol 92,584-588.

Sporn MD, Roberts AB, Shull JH, Ward JM, Sodek J (1983) Polypeptide transforming growth factors isolated from bovine sources and used for wound healing in vivo. Science 219:1329-1331.

Stenn KS (1981) Epibolin: A protein of human plasma that supports epithelial cell movement. Proc Natl Acad Sci USA 78,6907-6911.

Takashima A, Grinnell F (1985) Fibronectin-mediated keratinocyte migration and initiation of fibronectin receptor function in vitro. J. Invest Dermatol. 85:304-308.

Taylor, J.R., Halprin, K.M., Levine, V. and Woodyard, C. (1983) Effects of methotrexate in vitro on epidermal cell proliferation. British J Dermatol 108,45-61.

Viljanto J (1964) Biochemical basis of tensile strength in wound healing: An experimental study with viscose cellulose sponges in rats. Acta Chir Scand (Suppl) 333:1-10.

Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin C-H, Huang JS, Devel TF. 1983 Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304:35-39.

Westermark B, Heldin C-H, Ek B, Johnsson A, Mellstrom K, Nister M, Wasteson A (1983) Biochemistry of platelet-derived growth facotr. In: Growth and Maturation Factors (Guroff G, ed.), John Wiley and Sons, New York, pp. 73-115.

Winter GD (1972) Epidermal regeneration studied in the domestic pig. In: Epidermal Wound Healing (Maibach HI and Rovee DT, eds.) Year Book Medical Publishers, Inc., Chicago, pp. 71-112.

Yamada, K.M. and Olden, K. (1978) Fibronectins--adhesive glycoproteins of cell surface and blood. Nature (London) 274,179-184.

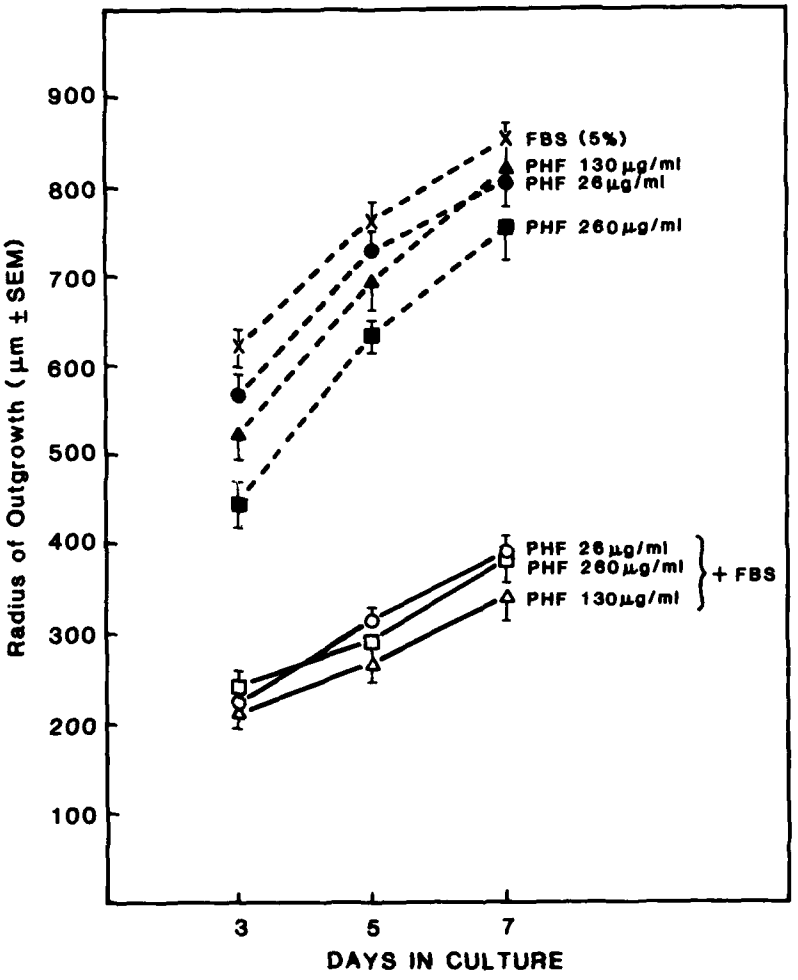

Figure 1: Extent of Epidermal Cell Outgrowth from Explants.

Repeated measures analysis of variance indicated that there was a dose-dependent effect on outgrowth with PHF alone. Addition of PHF and FBS together results in slower outgrowth that did not differ with the concentrations of PHF.

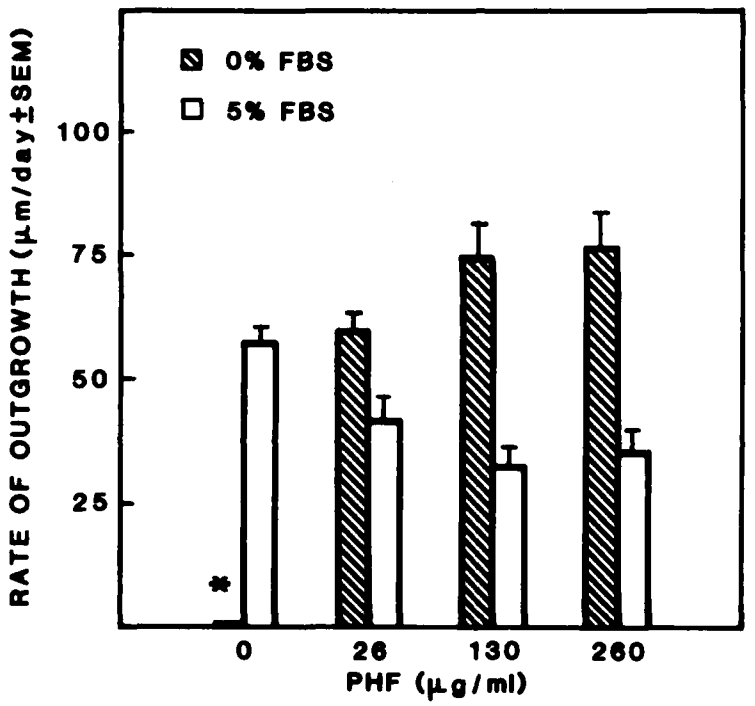

Figure 2: Differences in Outgrowth Rates Between Days 3 and 7.

The asterisk (*) marks the negative control group which was nonviabile; although 2/120 explants exhibited limited outgrowth, this was considered to be an artifact possibly due to residual nutrients within the explant tissue.

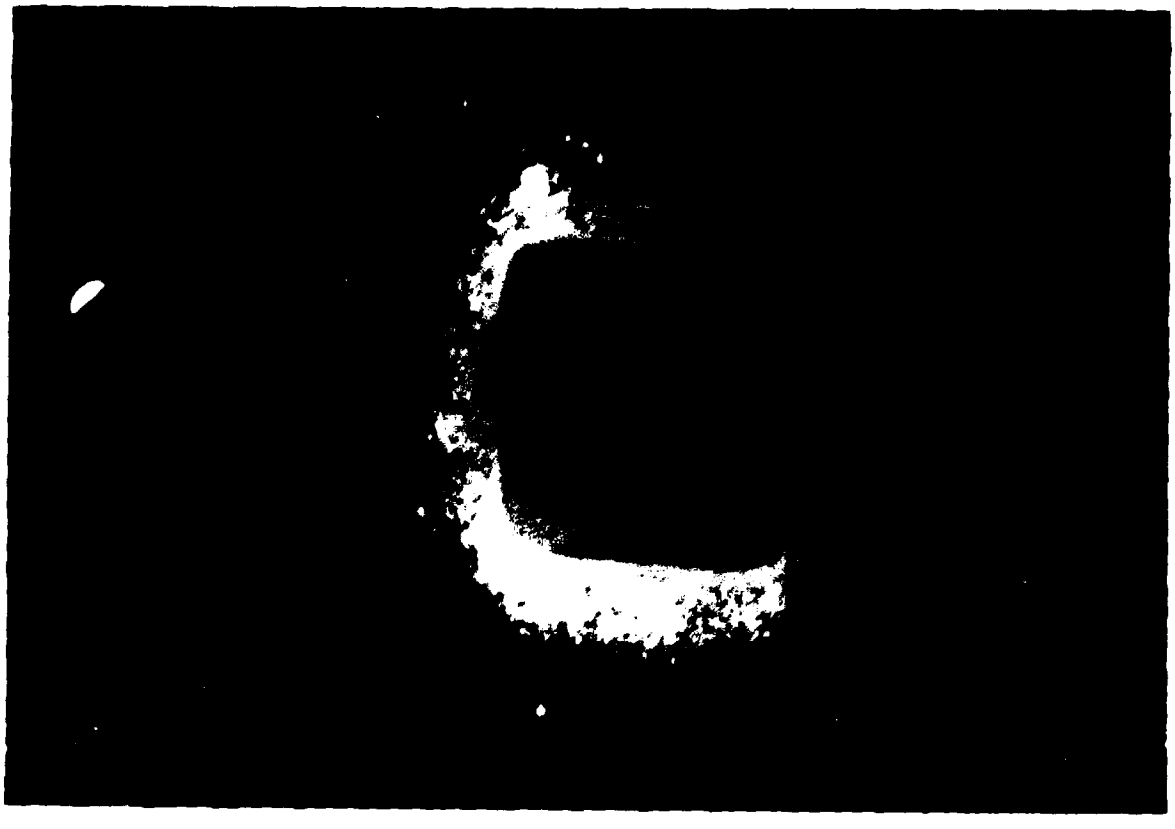

Figure 3A: Explant (Day 7) Cultured in 26 ug/ml PHF Under Dark Field Microscopy (31.2X).

The original explant (square piece of skin) is seen surrounded by the epidermal sheet (outgrowth). Note gradation in color of epidermal sheet indicating gradual thinning to a monolayer at the growing (outer) edge.

Figure 3B: Same Explant as in Fig 3A Shown in Phase Contrast at 125X.

Note thick, multilayered sheet near the explant (left) tapering to monolayer of "stretched" cells at the growing edge (right).

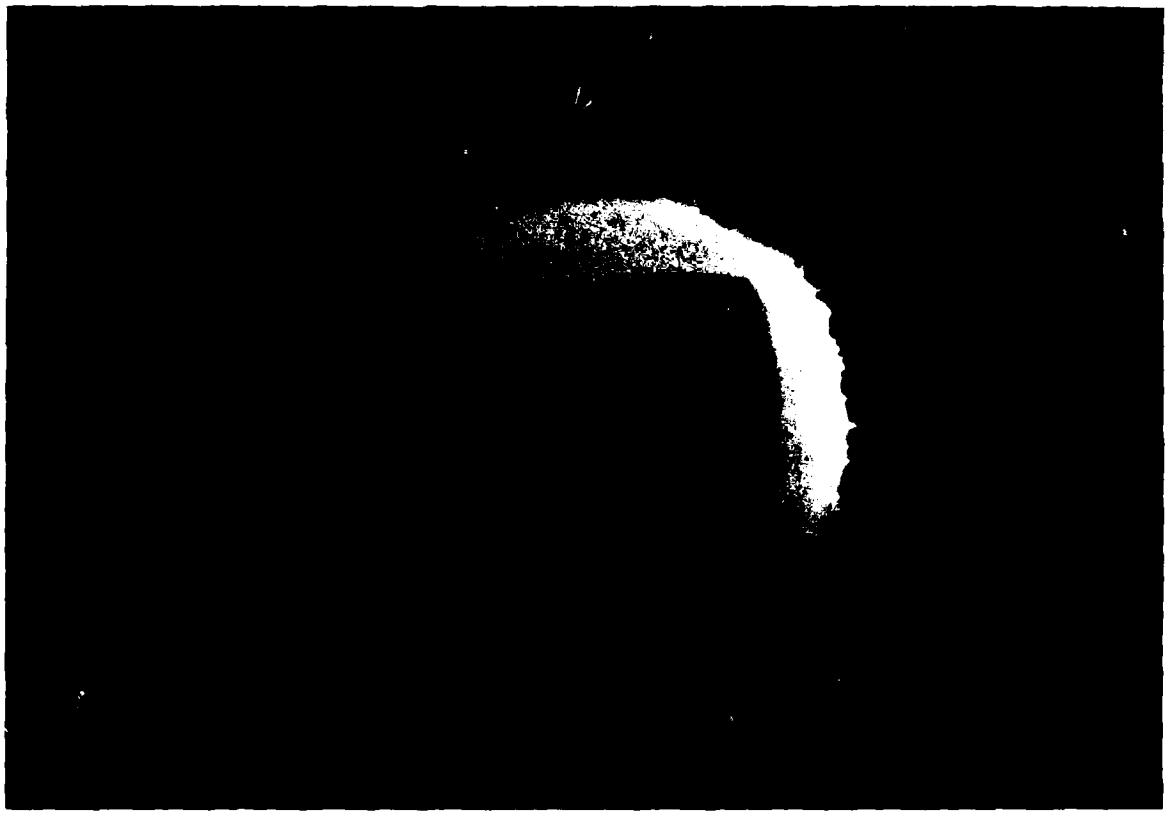

Figure 3C: Explant (Day 7) Cultured in 130 ug/ml PHF + 5% FBS Under Dark Field Microscopy (31.2X).

Note uniformly thick epidermal sheet.

Figure 3D: Same Explant as in Fig 3C Shown in Phase Contrast at 125X.

Note absence of flattened and "stretched" cells at growing edge (right). All the cells are rounded and piled up.

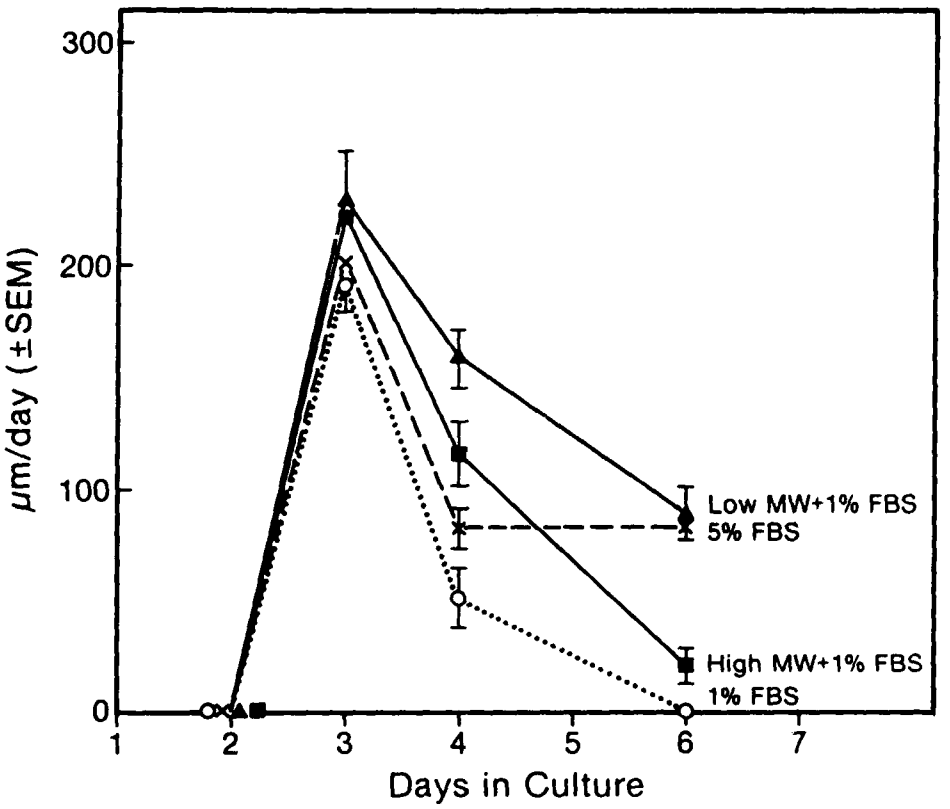

Figure 4: Rates of Outgrowth with PHF Fractions.

Fractions from ultrafiltration were tested for their ability to support explant outgrowth with 1% FBS. The Low Molecular Weight Fraction (<30K) appears to contain the "stimulatory" growth factor which produced a higher rate of outgrowth in the mitotic phase of culture (Days 4-6).

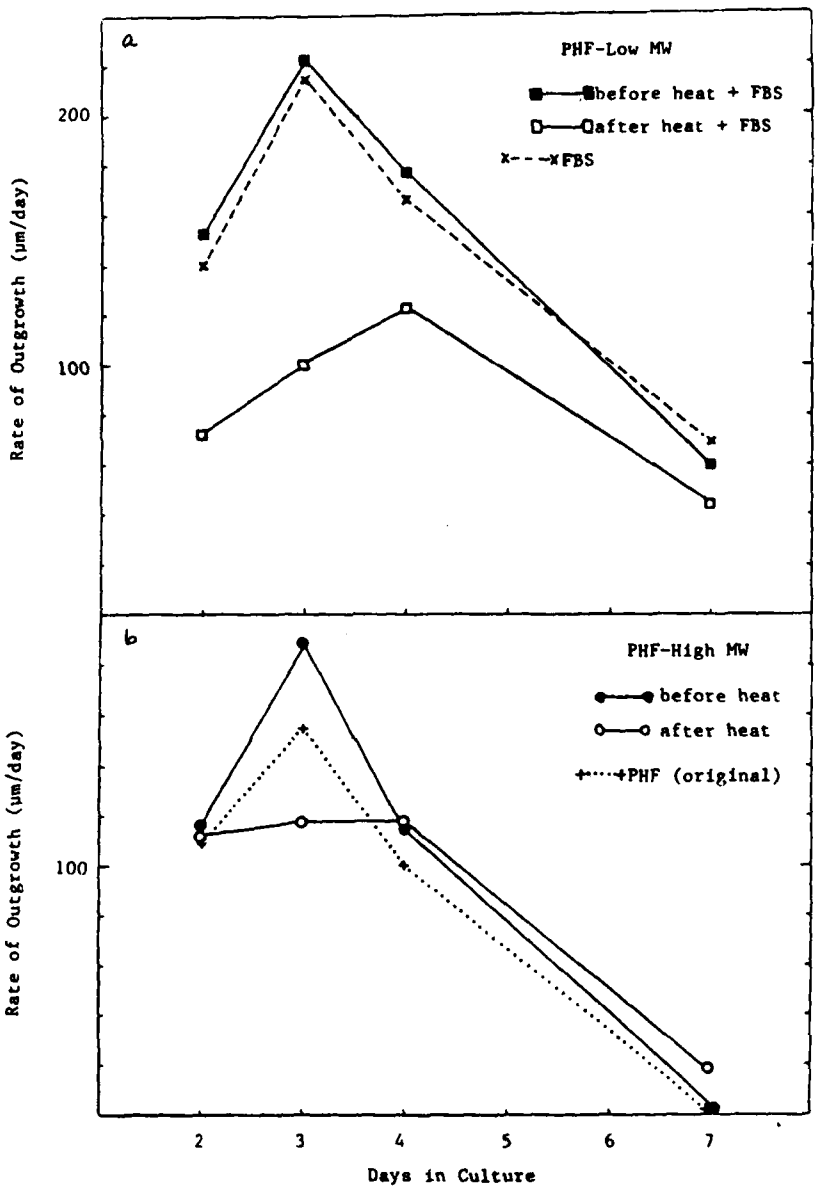

Figure 5: The Effects of Heat on the Stimulatory Activity of PHF High Molecular Weight and Low Molecular Weight Fractions.

Heat inactivation of PHF Low MW fraction, shown in Fig 5a, produced a much lower rate of outgrowth on Days 2-4. Heat inactivated PHF High MW, shown in Fig 5b, showed a lower rate of outgrowth on Day 3. The decreased rates of outgrowth suggest loss of stimulatory activity.

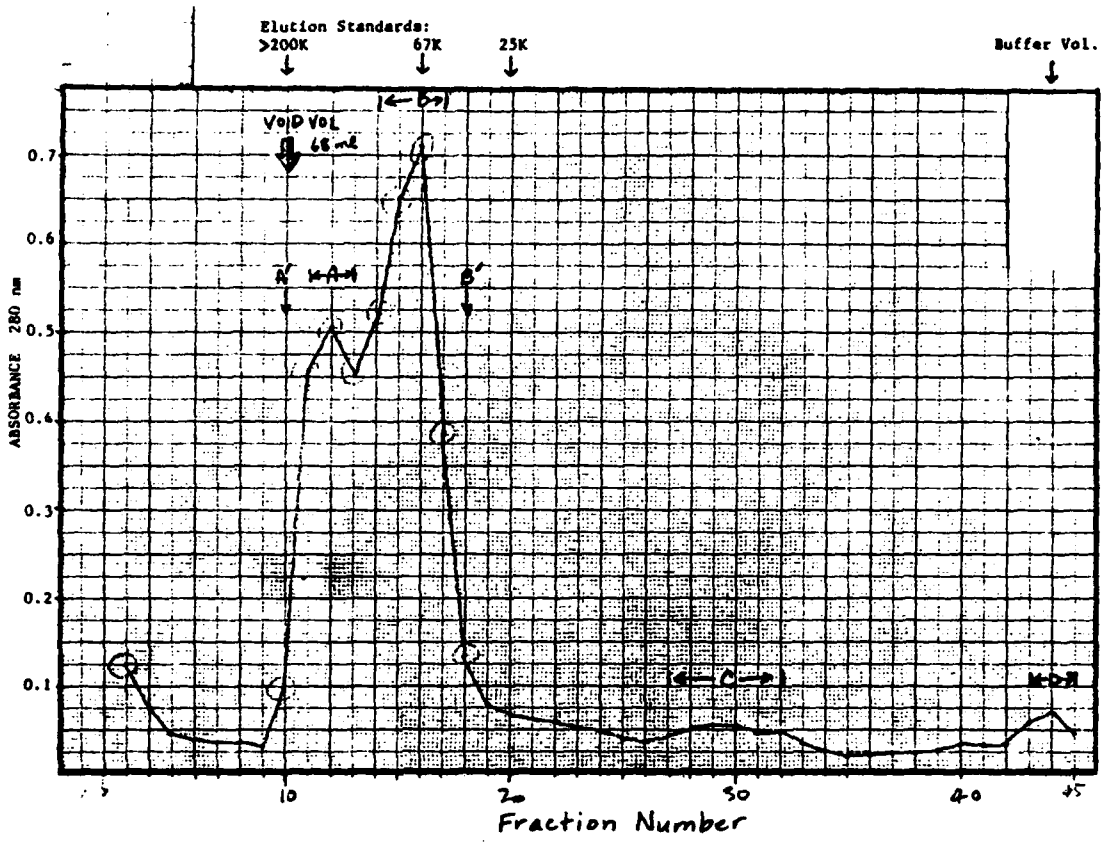

Figure 6: Separation of Platelet Homogenate Fraction (PHF) by Gel Filtration.

The sample, 7 ml PHF (5 mg/ml), was applied to a Sephacryl-200 column (1.6 x 67 cm) and eluted with platelet incubation medium at a flow rate of 17.5 ml/h. Fractions were collected (5.8 ml/tube) and the absorbance measured at 280 nm. Four peaks were obtained and the respective tubes were pooled, concentrated and assayed for activity with explants. In this experiment, none of the pooled fractions demonstrated activity.

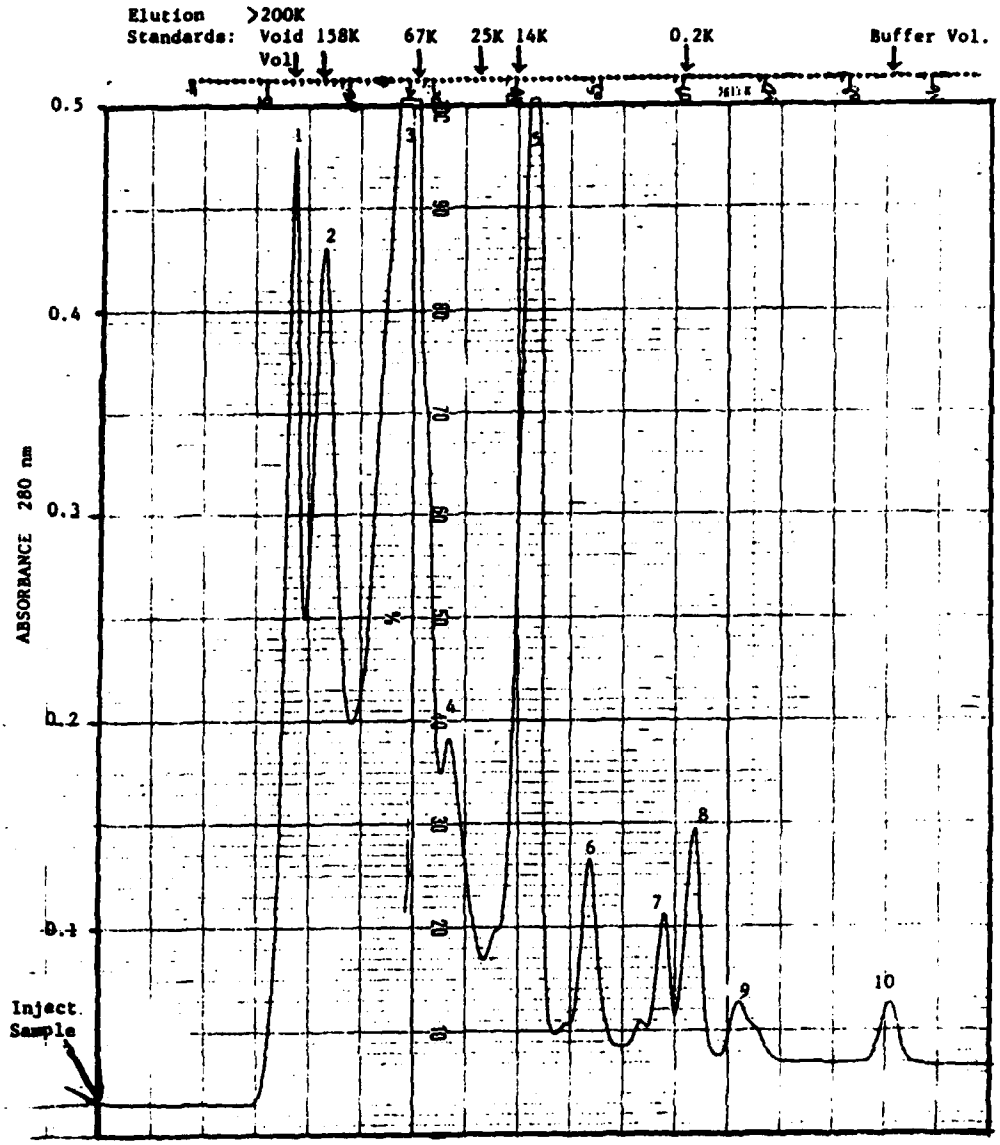

Figure 7: Separation of Platelet Homogenate Fraction (PHF) by FPLC Gel Filtration.

The sample of 0.5 ml PHF (7 mg/ml), was applied to a prepacked Superose-12 FPLC column (Pharmacia) and eluted with platelet incubation medium at a flow rate of 30 ml/h. Fractions were collected (0.5 ml/tube) and the absorbance measured at 280 nm. Ten peaks were obtained and the respective tubes were pooled and concentrated.

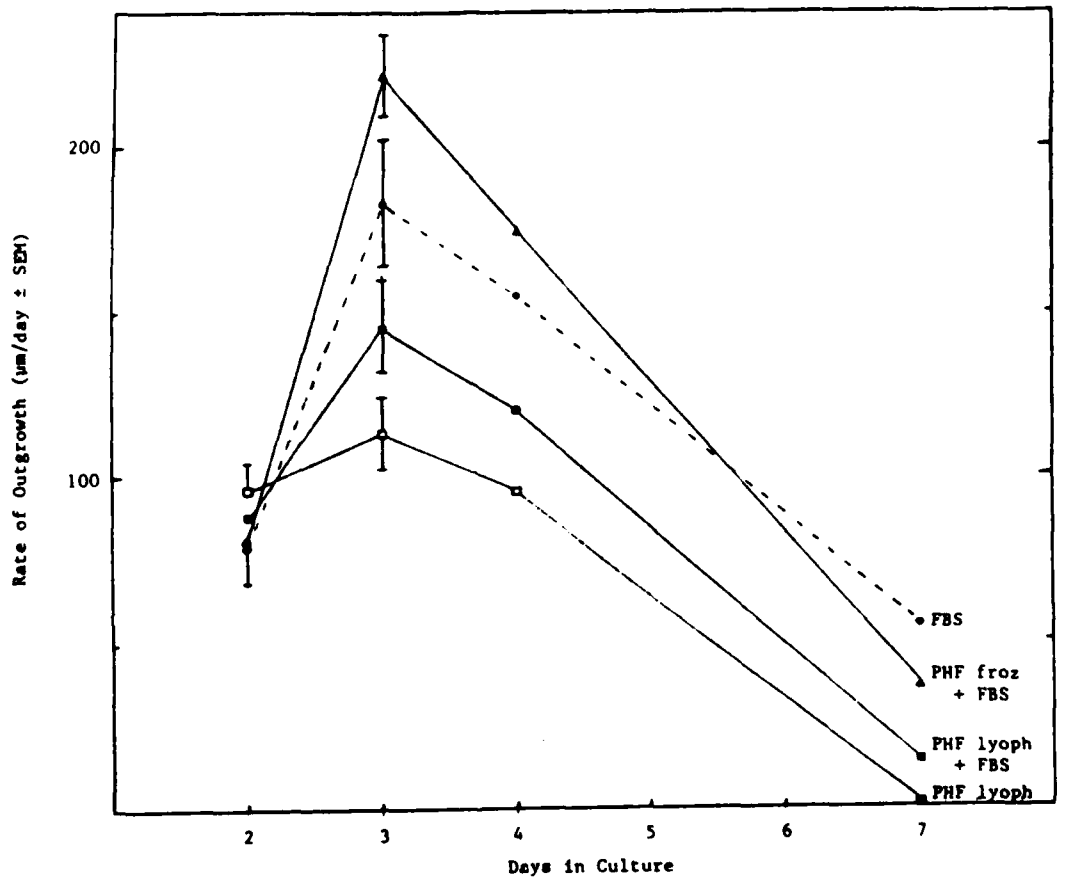

Figure 8: Rate of Outgrowth with PHF Preparations.

PHF preparations were stored under various conditions: frozen at -20°C or flash frozen (-200°C) and lyophilized. These preparations were tested for activity in the explant cultures. The rates of outgrowth indicate that there was some loss of activity in the lyophilized fraction but not the frozen fraction.

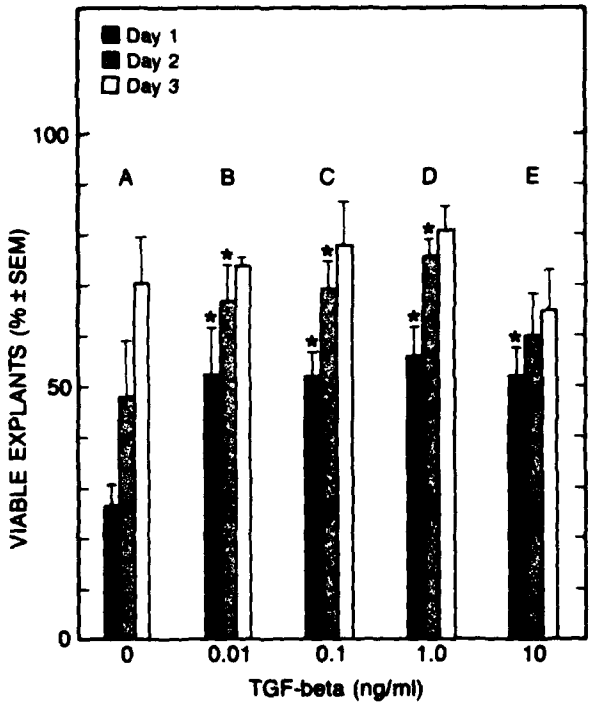

Figure 9a: Explant Viability with Transforming Growth Factor-beta.

The graph shows explant viability with 1% FBS Control (panel A) and 1% FBS plus various concentrations of TGF-beta (panels B-E). The bars indicate the percentage of viable (growing) explants on Days 1, 2 and 3 of culture. The asterisk (*) indicates the values that are different from the control (panel A) for each day, $p < 0.05$.

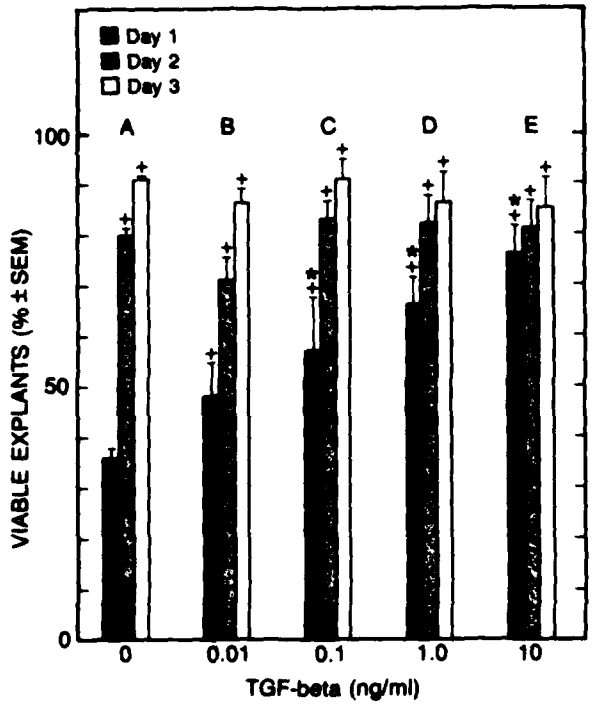

Figure 9b: Explant Viability with Transforming Growth Factor-beta and Epidermal Growth Factor.

This graph shows explant viability with 1% FBS plus 5 ng/ml EGF (panel A) and 1% FBS plus 5 ng/ml EGF plus various concentrations of TGF-beta (panels B-E). The series of treatments was evaluated in the same experiments as the series described in Fig 9a so that the results can be compared directly. The bars indicate the percentage of viable (growing) explants on Days 1, 2 and 3 of culture. The plus sign (+) indicates the values that are different from 1% FBS alone (Figure 9a, panel A), the asterisk (*) indicates the values that are different from FBS plus EGF (Figure 9b, panel A), $p < 0.05$.

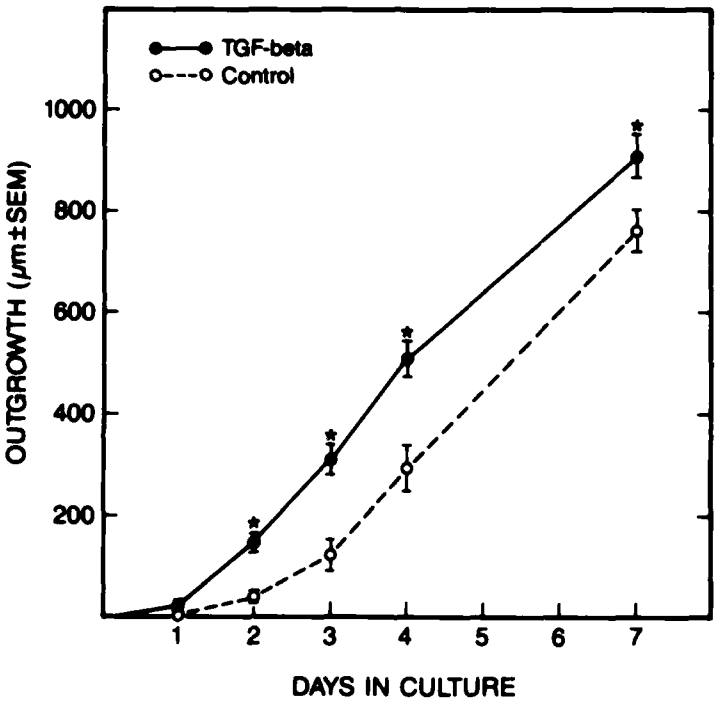

Figure 10a: Epidermal Outgrowth with Transforming Growth Factor-beta.

The curves show epidermal cell outgrowth with 1% FBS control and with 1% FBS plus 1.0 ng/ml TGF-beta. Outgrowth with TGF-beta was greater than the control on Days 2, 3, 4 and 7, $p < 0.05$, as indicated by an asterisk (*); however, there was no additional increases in the rate of outgrowth (slope) after Day 3.

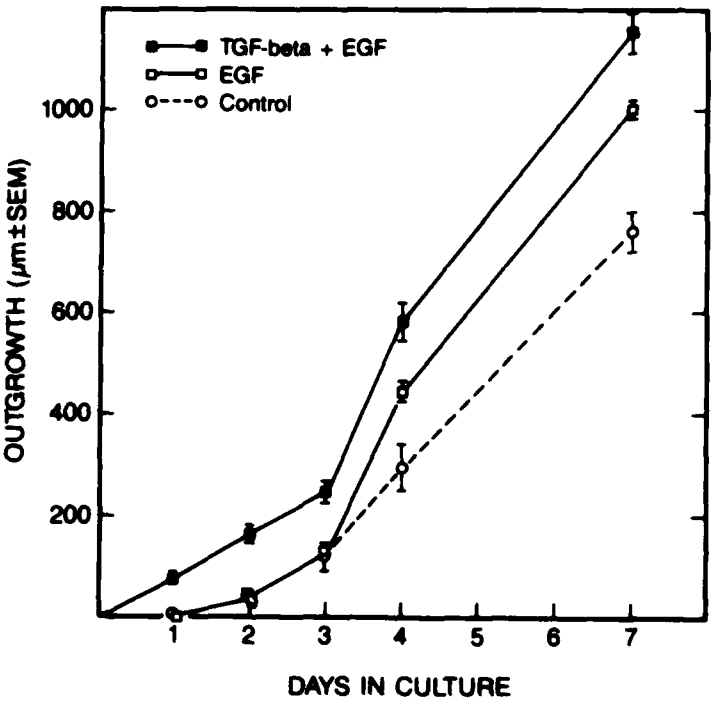

Figure 10b: Epidermal Outgrowth with Transforming Growth Factor-beta and Epidermal Growth Factor.

The curves show epidermal cell outgrowth with 1% FBS Control, with 1% FBS plus 5 ng/ml EGF and with 1% FBS plus 10 ng/ml TGF-beta and 5 ng/ml EGF. Outgrowth with EGF was greater than the control on Day 4 and 7 as indicated by a plus sign (+), $p < 0.05$. Outgrowth with TGF-beta plus EGF was greater than EGF alone on every day of evaluation as indicated by an asterisk (*), $p < 0.05$; however, there was no additional increase in the rate of outgrowth (slope) on Days 4 and 7.

Figure 11: Normal Skin Collagen Biosynthesis Assay: Effects of Repeated Sampling.

The Relative Collagen Biosynthesis was measured as described in the text. Results of daily sampling indicate a slight variation in the measurements which should not significantly affect the results obtained in wounds.

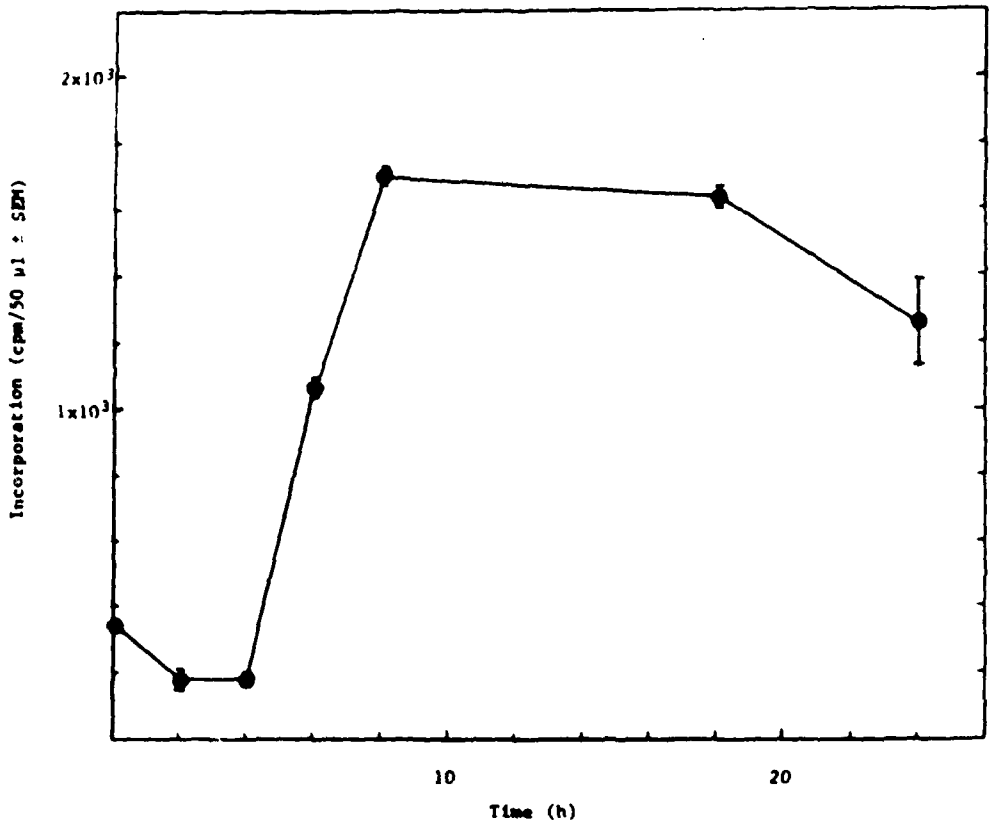

Figure 12: Time Course for the Incorporation of ^{14}C-proline by Minced Porcine Skin.

Significant incorporation was first observed at 6 hr, reached a plateau and was stable from 10-18 hr and decreased somewhat at 24 hr.

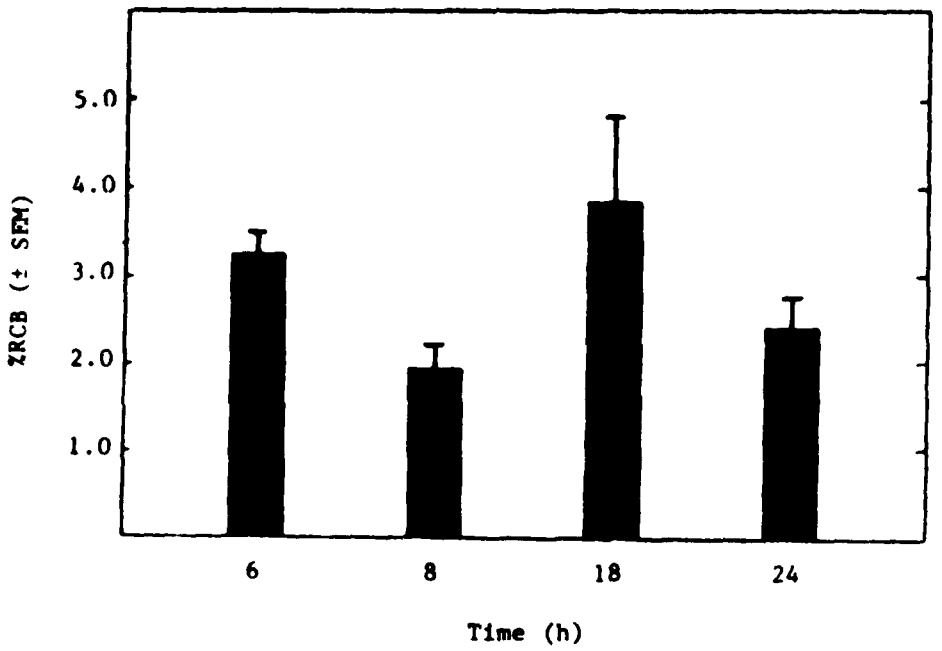

Figure 13: Time course for Relative Collagen Biosynthesis (%RCB) Measured in Porcine Skin.

The % RCB varied slightly and the relative maximum was measured at 18 hr.

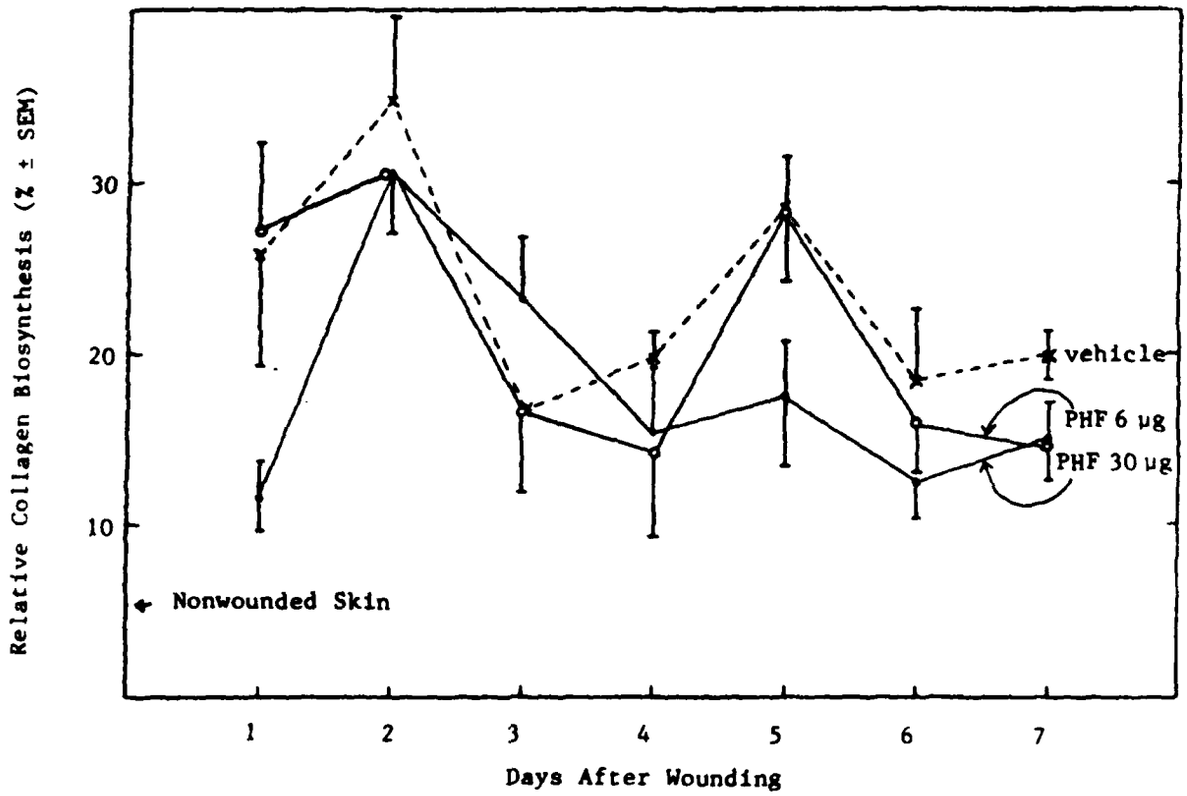

Figure 14: The Effects of Platelet Homogenate Fraction (PHF) in a Vehicle of Carboxymethylcellulose on Collagen Biosynthesis in Partial-thickness Wounds.

PHF at 6 ug/wound was not different from vehicle alone and PHF at 30 ug/wound produced lower %RCB on Days 1 and 5 compared with vehicle.

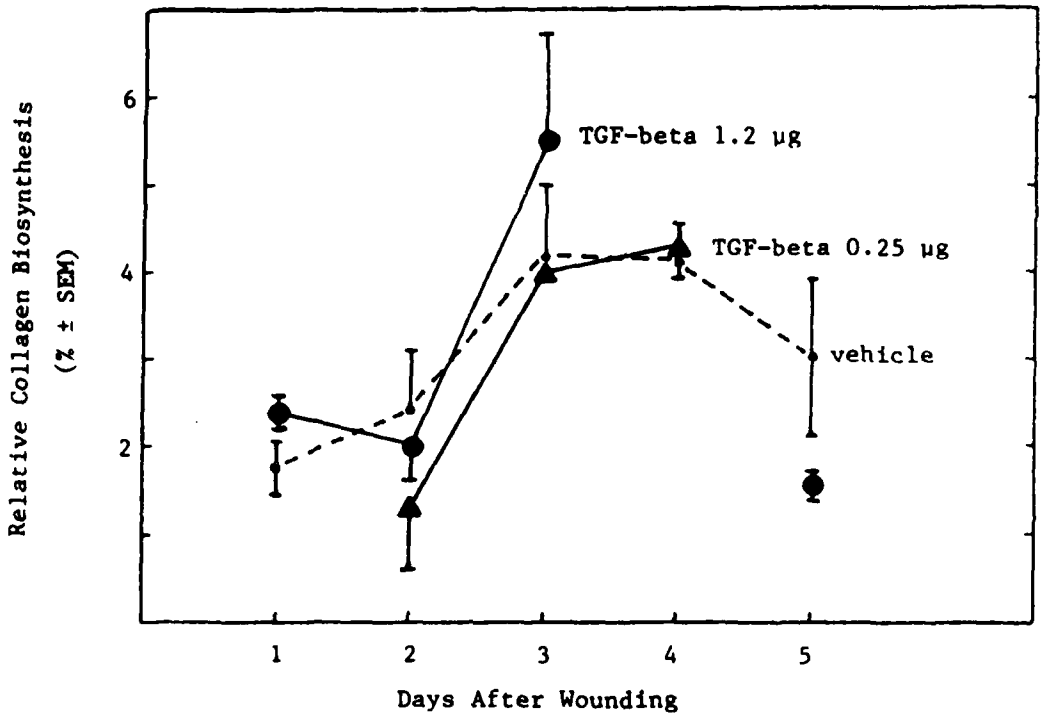

Figure 15: The effects of Transforming Growth Factor-beta (TGF-beta) on Collagen Biosynthesis in Partial-thickness Wounds.

Although incomplete, the data suggest that 1.2 ug/wound TGF-beta produced a higher %RCB on Day 3 compared to the other treatment groups.

Figure 16: Wound Treated with Platelet Fractions (100X).

Patient #1 was biopsied after daily treatment with her platelet fraction for 12 days. Note the stratum corneum (top), hyperplastic epithelium and granulation tissue.

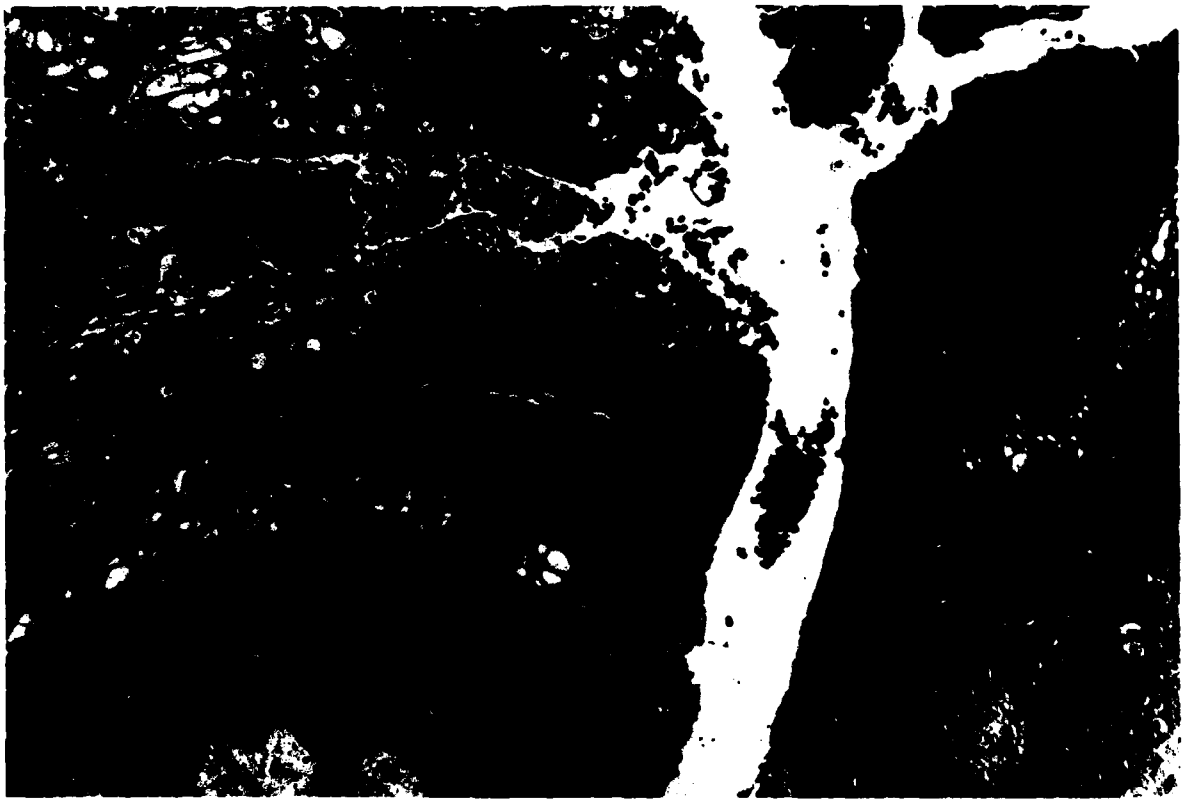

Figure 17a: Wound Treated with Platelet Homogenate Fractions (100X).

Patient 2# was biopsied after weekly treatment with her platelet fractions for three weeks. Note the thick epidermal layer with extensive keratinization and hyperplastic epidermis.

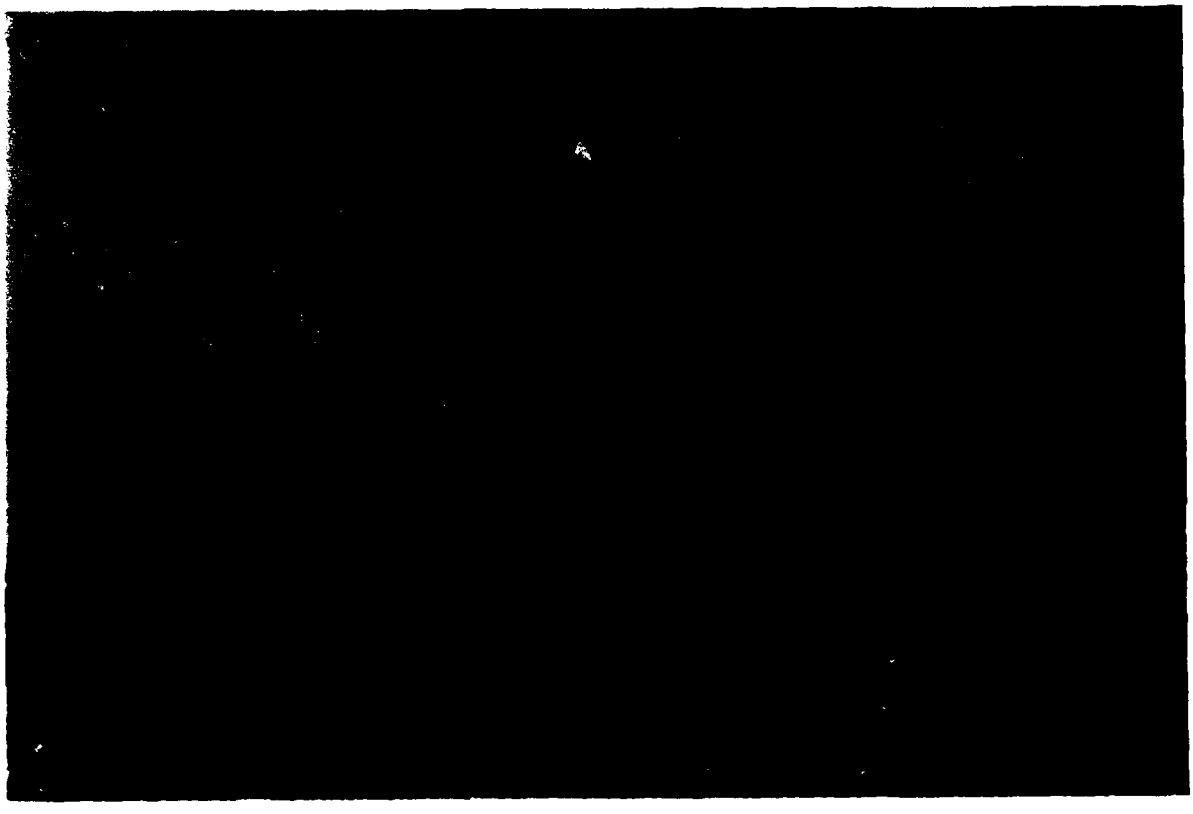

Figure 17b: Wound Treated with Vehicle (100X).

Patient #2 was biopsied at the same time as in Figure 2. Note the absence of a viable epidermal layer. There were areas of necrotic epidermis as shown in the upper left of this specimen.

TABLE 1: THE EFFECTS OF PLATELET HOMOGENATE FRACTION (PHF) AND FETAL BOVINE SERUM (FBS) ON EXPLANT OUTGROWTH.

0% FBS

Group No.	PHF (ug/ml)	Viability[a]	Extent of Outgrowth (um ± SEM) Day 3	Day 5	Day 7
1	0	2%	366 ± 79	516 ± 17	522 ± 11
2	26	69%	566 ± 24	728 ± 24	804 ± 25
3	130	45%	522 ± 26	692 ± 29	818 ± 32
4	260	55%	445 ± 26	633 ± 18	751 ± 33

5% FBS

Group No.	PHF (ug/ml)	Viability[a]	Extent of Outgrowth (um ± SEM) Day 3	Day 5	Day 7
5	0	67%	620 ± 21	761 ± 23	851 ± 20
6	26	30%	224 ± 13	314 ± 17	390 ± 20
7	130	26%	210 ± 14	265 ± 18	340 ± 24
8	260	37%	242 ± 17	290 ± 15	383 ± 24

[a] Viability = n/T x 100% (n = number of explants growing on Day 3 and T = total number of explants prepared, 120)

TABLE 2: DOSE-DEPENDENCY OF EXPLANT VIABILITY AND OUTGROWTH.

Treatment	Viability[a]	Rate of Outgrowth um/day (±SEM)	% Control[b]
PHF (ug/ml)			
0.026	0%	0	0%
0.26	0%	0	0%
2.6	58%	135 (±10)	41%
26	64%	290 (±20)	88%
130	70%	370 (±13)	109%
FBS (%)			
0	5%	165 (±1.6)	50%
5	84%	330 (±3.3)	100%

[a] See Table 1. (T = 40).

[b] Outgrowth is shown relative to the rate obtained with 5% FBS.

TABLE 3: THE EFFECT OF HEAT INACTIVATION ON PHF-SUPPORTED EXPLANT OUTGROWTH.

Experimental Conditions[a]	Viability	Rate of Outgrowth um/day (±SEM)	% Control[b]
RPMI 1640 alone (Negative Control)	25%	95 (±6)[d]	44%
RPMI 1640 + 5% FBS (Positive Control)	98%	216 (±9)	100%
RPMI 1640 + PHF			
(26 ug/ml)	78%	190 (±13)[c]	88%
(130 ug/ml)	70%	236 (±9)[c]	109%
RPMI 1640 + heated PHF			
100°C for 2 min (26 ug/ml)	58%	175 (±15)[d]	81%
100°C for 5 min (130 ug/ml)	15%	141 (±11)[d]	65%
RPMI 1640 + 5% FBS			
+ PHF (26 ug/ml)	98%	240 (±17)[c]	111%
+ heated PHF (26 ug/ml)			
100°C for 2 min	85%	264 (±15)d	122%
100°C for 5 min	70%	232 (±13)[c]	107%

[a] See Table 1. (T = 40)

[b] See Table 2

[c] Not significantly different from FBS control with Dunnett's procedure ($p > 0.05$)

[d] Significantly different from FBS control with Dunnett's procedure ($p < 0.05$)

TABLE 4: FRACTIONATION OF PHF BY MOLECULAR SIZE.

Fraction	Percent Total Protein	Viability[a]	Rate of Outgrowth um/day (±SEM)	% Control
PHF original	100%	64%	240 (±13)	100%
Low Molecular Weight (< 30 K)	10%[b]	20%	139 (±13)	58%
High Molecular Weight (> 30 K)	85%[b]	62%	220 (±11)	92%

[a] See Table 1. (T = 40)

[b] Total recovery in the two fractions = 95%

TABLE 5: VIABILITY WITH HEAT INACTIVATED PHF FRACTIONS.

Treatment	Viable Explants (%) N = 40 Day 1	Day 2	Day 3
PHF	18	32	48
PHF High MW	18	28	35
PHF High MW heat inactivated	18	18	38
0% FBS	0	12	20
1% FBS	30	48	58

TABLE 6: VIABILITY WITH PHF AFTER STORAGE.

Treatment	Viable Explants (%) N = 40 Day 2	Day 3
PHF		
1. fresh	60	80
2. quick frozen and lyophilized	74	83
3. frozen at -20°C	27	61
0% FBS	20	56
1% FBS	88	100

TABLE 7: THE EFFECT OF PLATELET-DERIVED GROWTH FACTOR (PDGF) ON EXPLANT OUTGROWTH.

PDGF[a] (units/ml)	Rate of Outgrowth 0% FBS	5% FBS um/day (±SEM)	% Control[b]
0	0	216 (±9)	100%
0.2	0	249 (±15)[c]	115%
1.0	0	260 (±9)[c]	120%
5.0	0	268 (±4)[c]	124%

[a] 1 unit/ml PDGF = the amount which evokes a response in fibroblasts equal to that of 5% FBS.

[b] See Table 2.

[c] By Dunnett's procedure PDGF plus 5% FBS produced greater outgrowth than FBS alone. The difference was significant for each of the three PDGF concentrations ($p < 0.05$).

TABLE 8: COMBINED EFFECTS OF TGF-beta, EGF AND PDGF ON EPIDERMAL CELL VIABILITY AND OUTGROWTH.

	Viability (% Growing Explants) N = 40			Extent of Outgrowth (um ± SEM)		
Treatment	Day 1	Day 2	Day 3	Day 2	Day 4	Day 7
Control	40	85	90	100 ± 12	398 ± 15	585 ± 19
PDGF	20*	89	89	53 ± 4*	365 ± 19	653 ± 32
TGF-beta+EGF	79	79	89	92 ± 11	420 ± 12	826 ± 25+
TGF-beta +EGF+PDGF	60*	80	95	109 ± 18	409 ± 11	793 ± 30+

* The values indicated were lower than the corresponding treatment without PDGF ($p < 0.05$).

+ The values indicated were higher than the control ($p < 0.05$), but not different from each other.

TABLE 9: THE EFFECT OF CYCLOHEXIMIDE ON EXPLANT VIABILITY AND OUTGROWTH

Treatment	cycloheximide (25 ug/ml)	Viability (% Growing Day 3) N = 40	Rate of Outgrowth (um/day ± SEM) Day 2	Day 4	Day 7
FBS (1%)	-	61	140(±34)	238(±39)	43(±15)
	+	0	0	0	0
PHF (26 ug/ml)	-	63	71(±12)	132(±16)	29(±6)
	+	0	0	0	0
TGF-beta (1.0 ng/ml) + FBS (1%)	-	81	257(±16)	153(±16)	21(±3)
	+	0	0	0	0
TGF-beta (1.0 ng/ml) + EGF (5 ng/ml) + FBS (1%)	-	86	178(±13)	205(±19)	55(±7.4)
	+	0	0	0	0

TABLE 10: THE EFFECT OF MONENSIN ON EXPLANT VIABILITY AND OUTGROWTH

Treatment	monensin (1×10^{-6} M)	Viability (% Growing Day 3) N = 40	Rate of Outgrowth (um/day ± SEM) Day 2	Day 4	Day 7
FBS (1%)	-	53	112(±16)	177(±25)	124(±13)
	+	79	89(±16)	150(±24)	127(±10)
PHF (26 ug/ml)	-	25	0	133(±16)	110(±9)
	+	27	0	133(±17)	43(±9)
TGF-beta (1.0 ng/ml) + FBS (1%)	-	100	57(±15)	178(±28)	170(±18)
	+	85	93(±10)	164(±32)	94(±10)
TGF-beta (1.0 ng/ml) + EGF (5 ng/ml)	-	68	50(±11)	162(±26)	185(±12)
+ FBS (1%)	+	100	35(±24)	143(±15)	54(±12)

TABLE 11: TREATMENT OF WOUNDS DURING DOSE/FREQUENCY RESPONSE STUDIES

Group No.	Treatment[a]
1	3×10^{10} pl/ml[b] in phosphate-buffered saline
2	3×10^{9} pl/ml in phosphate-buffered saline
3	3×10^{8} pl/ml in phosphate-buffered saline
4	vehicle - phosphate-buffered saline
5	control - untreated

[a] In each series pig 1 was treated daily, pig 2 was treated only on day of wounding.

[b] Platelets/ml; the platelets were activated in the second series with thrombin (20 units/ml), in the third series by homogenization.

AD-A182 169 DERMAL INFLUENCE ON EPIDERMAL RESURFACING DURING THE REPAIR OF SPLIT THICKNESS WOUNDS(U) PITTSBURGH UNIV PA P A HEBDA ET AL. JUN 86 DAMD17-82-C-2149 2/2

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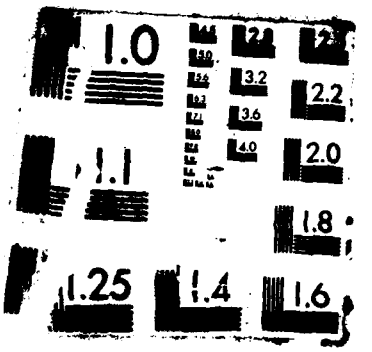
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1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0

TABLE 12: RATE OF EPIDERMAL WOUND HEALING WITH PLATELET HOMOGENATES.

Group	Treatment	HT_{50} [a]
I	3×10^{10} pl/ml	4.7 days
II	3×10^{9} pl/ml	5 days
III	3×10^{8} pl/ml	5 days
IV	vehicle	5 days
V	untreated	5 days

[a] Time at which 50% of the wounds were healed.

TABLE 13. THE EFFECT ON EPIDERMAL RESURFACING OF TOPICALLY APPLIED PLATELET HOMOGENATE

Group	Treatment	HT_{50}[a]
Active	3×10^{10} platelets/ml in PIM[b]	5.4 days
Vehicle	PIM	5.4 days
Untreated	---	5.4 days

[a] HT_{50} = Healing Time 50 - time required for 50% of the wounds to heal.

[b] PIM = Platelet Incubation Medium - 100 mM KCl, 50 Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4.

TABLE 14. THE EFFECT OF DERMAL COLLAGEN BIOSYNTHESIS OF TOPICALLY APPLIED PLATELET HOMOGENATE

Group	Treatment	Relative Collagen Biosynthesis[a] Relative Units (SEM)
Active	3×10^{10} platelets/ml in PIM[b]	1.68 (0.37)[c]
Vehicle	PIM	1.96 (0.52)[c]
Untreated	---	1.00

[a] The mean values from four animals of the % RCB on post-wounding Day 2 (the time during which collagen biosynthesis is stimulated) have been normalized to Untreated wounds to correct for animal to animal variation.

[b] PIM = Platelet Incubation Medium - 100 mM KCl, 50 Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4.

[c] Not significantly different ($p > 0.05$)

TABLE 15: EFFECTS OF PLATELET HOMOGENATE ON WOUND RE-EPITHELIALIZATION.

A. Study #1 6 hr of occlusion

Treatment	No. of Wounds Healed/No. of Wounds Evaluated				
	Day 3	Day 4	Day 5	Day 6	Day 7
Untreated	0/10 (0%)	1/10 (10%)	2/7 (29%)	6/10 (60%)	10/11 (91%)
Vehicle	0/9 (0%)	4/10 (40%)	6/9 (67%)	9/9 (100%)	8/8 (100%)
PHF	0/10 (0%)	0/10 (0%)	5/7 (72%)	9/9 (100%)	9/9 (100%)

B. Study #2 2 hr of occlusion

Treatment	No. of Wounds Healed/No. of Wounds Evaluated				
	Day 3	Day 4	Day 5	Day 6	Day 7
Untreated	0/10 (0%)	1/11 (0%)	8/9 (89%)	10/10 (100%)	7/7 (100%)
Vehicle	0/8 (0%)	9/10 (90%)	9/9 (100%)	8/9 (89%)	10/10 (100%)
PHF	0/9 (0%)	6/8 (75%)	10/10 (100%)	10/10 (100%)	7/7 (100%)

TABLE 16. THE EFFECT OF PLATELET HOMOGENATE FRACTION IN A VEHICLE OF CARBOXYMETHYL CELLULOSE ON RE-EPITHELIALIZATION IN PARTIAL-THICKNESS WOUNDS

Treatment	Percent Healed (N = 10 wounds from 2 animals) Day 3	Day 4	Day 5	Day 6	HT_{50} [a]
Vehicle[b] (2.5% carboxymethylcellulose covered with adhesive occlusive dressing, Tegaderm®)	0	30	70	100	4.5
PHF					
6 ug/wound	20	20	100	100	4.4
30 ug/wound	20	40	100	100	4.2
Untreated (air-exposed control)	0	50	90	100	4.0

[a] HT_{50} = Healing Time 50 - time required for 50% of the wounds to heal.

[b] The vehicle became very adherent to the wounds and with daily removal and re-application there was the possibility of rewounding, accounting for the HT_{50} values higher than the untreated, air-exposed control group.

TABLE 17. THE EFFECT OF TRANSFORMING GROWTH FACTOR-beta ON RE-EPITHELIALIZATION IN PARTIAL-THICKNESS WOUNDS

Treatment	Percent Healed (N = 10 wounds from 2 animals) Day 3	Day 4	Day 5	HT_{50} [a]
Vehicle (petrolatum)	0	50	100	4.0
TGF-beta				
0.25 ug/wound	0	64	100	3.8
1.2 ug/wound	0	20	100	4.4
Untreated (air-exposed control)	0	0	100	4.5

[a] HT_{50} = Healing Time 50 - time required for 50% of the wounds to heal.

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